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The Role of Serum Enterolactone in Lipid Peroxidation and Cardiovascular Disease

Doctoral dissertation

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ABSTRACT

Dietary polyphenols have attracted scientific attention due to the epidemiological evidence showing significant associations between polyphenol rich diet and decreased risk of cardiovascular diseases (CVD). According to their carbon skeleton, polyphenols can be classified to phenolic acids, flavonoids, stilbenes and lignans. The chemical structure of polyphenols makes them potential antioxidants and this idea has been supported by numerous in vitro studies. The current knowledge on the pathogenesis of atherosclerosis emphasises the importance of low density lipoprotein (LDL) oxidation, which can be inhibited by antioxidants. Enterolactone is formed in the metabolism of many dietary lignans by the interaction of intestinal bacteria. The development of immunological detection methods has enabled the analysis of serum enterolactone concentration in population studies. The aim of this work was to study the role of serum enterolactone concentration with regard to cardiovascular health and lipid peroxidation in middle aged men. We also investigated the possibility of increasing serum enterolactone level by supplementing phloem powder enriched rye bread.

We examined the association of serum enterolactone concentration with the risk of acute coronary events in a case-control design, nested in a prospective cohort study, the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. Serum enterolactone concentration was measured from baseline samples for 167 men who had an acute coronary event during an average follow-up of 7.7 years and for 167 matched controls. The risk of acute coronary events was lower among the men at the higher end of the serum enterolactone distribution compared with the men with lower concentrations. In continuation serum enterolactone concentration was measured in the rest of the men in the cohort, whose baseline serum sample was available. Consequently the risk for all-cause and coronary heart disease (CHD) and CVD-related death was studied in 1889 men free of CVD history. The multivariate analysis showed significant associations between elevated serum enterolactone concentration and reduced risk of CHD-related, CVD-related and all-cause death.

In a subset of the Antioxidant Supplementation in the Atherosclerosis Prevention (ASAP) Study we investigated the association between serum enterolactone concentration and in vivo lipid peroxidation, as measured by plasma F2-isoprostanes. We found that serum enterolactone concentration had an inverse correlation with F2-isoprostanes and that serum enterolactone concentration was one of the strongest determinants of lipid peroxidation. To study the sensitivity of serum enterolactone concentration in detecting changes in dietary lignans and to investigate the possibility of using phloem supplementation in rye bread to increase serum enterolactone level, a randomised double blind supplementation study was performed. Seventy-five men were randomised to three study groups receiving 70 g of one of the three rye breads, containing different amounts of phloem powder. Significant changes in serum enterolactone concentration were observed among the participants following the supplementation but due to large individual differences in enterolactone production, clear dose-response effect could not be seen.

This work suggests that serum enterolactone might have a role with regard to cardiovascular health in Finland, at least in men. The presented associations of serum enterolactone concentration with CVD and lipid peroxidation can also reflect a role of dietary lignans or other associated compounds. Our findings give further support to the view that plant-dominated diet is favourable for cardiovascular health.

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Medical Subject Headings: F2-isoprostanes/blood; lipid peroxidation; antioxidants; free radicals; lignans; dietary fibers; nutrition; cardiovascular diseases; coronary disease; risk factors; human; male; randomized controlled trials; double-blind method; follow-up studies; case-control studies; cross-sectional studies; prospective studies
YHTEENVETO


Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) -tutkimuksessa (100 riehän otoksessa) selvittimme seurumin enterolaktonpitoisuuden yhteyttä lipidien hapettumiseen mittaamalla lipidien hapettumista plasman F<sub>2</sub>-isoprostaanipitoisuudella. Matalan seurumin enterolaktonpitoisuuden todettiin olevan yhteydessä kohonneeseen plasman F<sub>2</sub>-isoprostaanipitoisuuteen. Näin lipidien hapettuminen oli sitä runsaampaa, mitä pienempi oli tutkittavan seurumin enterolaktonpitoisuus. Viimeisessä selvittimme kaksios-sokkotutkimuksella ravinnon lignaanien vaikutusta seurumin enterolaktonpitoisuteen sekä mahdollisuutta nostaa sen pitoisuutta verenkierrossa antamalla petteilupäät, joka sisältää puikkeuksellisen runsaasti lignaania. Tutkimukseen osallistui 75 vapaaehtoista miestä, jotka soivat neljän viikon ajan päivittäin 70 grammmaa yhtä kolmosa tutkimusleivästä. Kahdessa tutkimusleivässä oli osa niinjauhoista korvattua eri määrällä pettiujauhoa ja kolmas tutkimusleijon oli tavallinen ripukarp. Tutkimusleijojen pettiujauhon määrä vaikutti selvästi seurumin enterolaktonpitoisuuteen, mutta tarkoittavat reagoivat hyvin yksilöllisesti ravinnon suureen lignaanimäärän eikä suurista toinen lignaanien ravinnossa harikoilla nostanut seurumin enterolaktonpitoisuutta.

Tämän väitöskirjan perusteella voidaan sanoa, että seurumin enterolaktonilla saattaa olla merkitystä suomalaisten miesten sydän- ja verisuonisairauksien ehkäisyssä. Ravinnon lignaanit sekä myös muut ravinnon polyfenoliset yhdisteet voivat olla tärkeitä antioksidanteja. Tutkimus tuo myös esille suoliston hakteerifloona keskeisiin tehtäviin niiden yhdisteiden metaboliossa, joka saattaa selittää yksilöllisen vasteen ravinnon lignaanille. Tämän väitöskirjatuotkimuksen tulokset tukevat aikaisempia tutkimuksia, joissa runsaasti kasvinsä ja kuitupitoisia viljatuotteita sisältävän ravinnon on todettu suojavasta sydän- ja verisuonaitaudelliita.
To Pirjo-Riitta and Heikki,
whose love gave me the chance.

Markus,
for excelling as a brother.

Jaakko,
for wanting to share the true challenge,
life.
Fine writers should split hairs together, and sit side by side, like friendly apes, to pick the fleas from each other’s fur.

LOGAN PEARSELL SMITH
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Meri Vanharanta
ABBREVIATIONS

AMI  Acute myocardial infarction
ASAP  Antioxidant Supplementation in the Atherosclerosis Prevention Study
BMI  Body mass index
CI  Confidence interval
CHD  Coronary heart disease
CVD  Cardiovascular diseases
DNA  Deoxyribonucleic acid
EDTA  Ethylenediamine tetraacetic acid
e.g. exemplia gratia (for example)
ELISA  Enzyme linked immunosorbent assay
EPIC  European Prospective study Into Cancer and nutrition
ECG  Electrocardiogram
GC-MS  Gas chromatography-mass spectrometry
HDL  High density lipoprotein
HPLC  High-performance liquid chromatography
ICD  International Classification of Diseases
IGF-I  Plasma insulin-like growth factor I
KIHD  Kuopio Ischaemic Heart Disease Risk Factor Study
HP  High phloem group
LDL  Low density lipoprotein
LP  Low phloem group
MDA  Malondialdehyde
MONICA  Monitoring of Trends and Determinants of Cardiovascular Disease
OR  Odds ratio
PUFA  Polyunsaturated fatty acids
RNS  Reactive nitrogen species
ROS  Reactive oxygen species
RR  Relative risk
SD  Standard deviation
SPSS  Statistical Package for the Social Science software
TBARS  Thiobarbituric acid test (thiobarbituric acid reactive substances)
tHcy  Total homocysteine
TR-FIA  Time-resolved fluoroimmunoassay
WHO  World Health Organization
LIST OF ORIGINAL PUBLICATIONS


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1 INTRODUCTION

Coronary heart disease (CHD) and other cardiovascular diseases (CVD), including stroke, are the leading cause of death in most industrialised countries. In Finland CVD remain as the top public health problem and circulatory deaths account for almost half of all deaths in the country. The trend is however downwards and since the late 1960’s the age-standardised CHD mortality and morbidity have steadily declined although the decline has slowed down in recent years. Ageing of the population also means that the numbers affected and requiring treatment for CHD will increase. Today in Finland CHD mortality among women is lower than in most European countries. Unfortunately Finnish men are still an example of a high CHD mortality population compared with other western countries. This stands, regardless of the 50 % decline in CHD mortality rate since the end of 1960’s, which has extended their lead in life expectancy as compared with their peers in neighbouring Baltic countries and Russia. Mostly due to circulatory diseases, regional differences in mortality within Finland have been apparent throughout the years, where eastern and northeastern parts have been distinguished by a much higher incidence of CHD (1).

Of the two areas in Finland that participated in the Seven Countries Study published in the late 1970’s, the province of North Karelia, from the northeastern part of Finland, stood out from all the other regions as being most dramatically affected by the burden of ischaemic heart disease (2). In light of the most essential risk factors for CVD, which are blood lipids, blood pressure and smoking (3), the exceptionally high mortality rates of young North Karelian men could be understood. More than half of the men were smokers and the highest levels of average serum cholesterol and blood pressure ever measured in a population were found among them. Fortunately, the prevalence of risk factors has shown significant improvement not only in North-Karelia but also nation wide over the past 25 years (2). It has been estimated that of the 55 % age-adjusted decline in CVD mortality among men in Finland between 1972 and 1992, about three-quarters can be explained by changes in these three risk factors. The most important of these changes is suggested to be the decrease in serum total cholesterol, which again can be explained by the dietary shift from saturated fat towards polyunsaturated fat and other nutritional factors together (4).

Regarding the role of nutrition in the prevention of CVD, the importance of dietary fat, through its impact on serum cholesterol level and on obesity in many cases, is rather well established although, as always the debate is still ongoing (5, 6). The epidemiological
evidence concerning carbohydrates is inconclusive but suggesting a weak inverse association with CHD (7, 8). The type, rather than the amount of dietary carbohydrates is likely to be a more important factor in determining CHD risk. The analysis of carbohydrate intake and glycemic index by Liu and co-workers from the Nurses’ Health Study indicate that high dietary glycemic load from refined carbohydrates increases the risk of CHD (9). Fibre content of food item is one of the core elements affecting its glycemic load. However, considering the abundance of epidemiological studies, which associate dietary fibre and whole grains with reduced risk of CHD (10), the protective effect of low glycemic load could have been anticipated. Few years ago the evidence on the cardio-protective effects of fruit and vegetable intake were reviewed by Ness and Powles (11) including results from many of the recent major cohort studies (10, 12-14) with the conclusion that a protective effect is apparent.

Oxidative modification of low density lipoprotein (LDL) is thought to be a prerequisite for cellular accumulation of cholesterol, which is a key contributor to the onset of atherosclerosis (15). Dietary antioxidants are assumed to prevent this damage and thus the atherosclerotic progression. Halliwell has questioned whether the protective effect of the diets rich in fibre, whole grain and fruit and vegetables are due, in whole or in part, to antioxidant mechanisms and also doubted whether these diets actually decrease oxidative damage in vivo (16). These questions are currently very relevant and still unravelled despite of the continuous scientific effort in the field. In search for the possible antioxidative or distinctly beneficial constituents in these diets rich in plant foods, combinations of vitamins, minerals but also plant enzymes, hormones and other phytochemicals have been considered (17). The most optimistic expectations during the last years have been set on the CVD protective potential of vitamins C, E and folate (18). Characteristic of the research path for many of the vitamins is the rather promising evidence from observational studies (19-21) but then several inconsistencies in showing the protective effect in supplementation trials (22-24). The results from these large-scale interventions have generated plenty of discussion, in which the difficulty to contemplate the reasons for the inconsistent results is obvious (25-28).

In observational studies one can search for the protective constituents against CHD in food by statistically adjusting the models with the potential nutrients. Liu and co-workers, reporting from the Nurses’ Health Study, decided to adjust whole grain intake for dietary fibre, folate, vitamin B-6 and vitamin E, despite of their positive association with whole grain. The authors concluded that to a large degree, the observed risk reduction with high intake of whole grains was not explained by these constituents thought to be protective (29). Overall there seems to exist evidence from both experimental as well as from observational studies
that even the abundance of vitamins in these foods does not fully explain the beneficial effect of plant-dominated diets. This notion has led to the investigation of other, less known individual components in fruits, vegetables and whole grains such as polyphenols, mainly flavonoids and lignans.

Of the thousands of compounds in the flavonoid group, analysis results on their quantity in foodstuffs are available only for a limited number of flavonoids. Several but not all prospective follow-up studies have shown high flavonoid intake (based on the data on three flavonols and two flavones) to be associated with reduced the risk of CVD (30-33). Flavonoids form a diverse group of compounds and characteristically carry two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (34). They are known as plant pigments and thus considered as responsible for the display of colour in many fruits and vegetables. Lignans share the polyphenol structure of flavonoids with their two phenol groups. Nonetheless, they are often attached to the fibre fraction and woody parts in plants and thus associated rather with bitter taste than with attractive colour! Databases for two dietary lignans, matairesinol and secoisolariciresinol have become available recently (35). The early discovery of enterolactone, a considerably stable metabolic end product of the mentioned and some other plant lignans, has enabled the development of quantitative detection methods in biological fluids.

As there are no previous studies on lignans and CVD and CVD-related mortality, these issues were examined in the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. Additionally we studied the association between serum enterolactone concentration and lipid peroxidation in the Antioxidant Supplementation in the Atherosclerosis Prevention (ASAP) Study and investigated the possibility of using phloem supplementation in rye bread to increase serum enterolactone concentration in a clinical trial.
2 REVIEW OF THE LITERATURE

2.1 Pathogenesis of CVD

2.1.1 Introduction to oxidative stress

Oxidative modification particularly of lipids is believed to be a key element in the aetiology of CVD (36). In this pathogenic process the molecular oxygen that gives rise to highly reactive free radicals has a central role. Free radicals, such as the reactive oxygen (ROS) and nitrogen (RNS) species include singlet oxygen, the superoxide anion radical, the peroxide anion, the hydroxyl radical, nitrogen oxides and peroxynitrite among others (37). These reactive oxygen species are generated during normal metabolic conditions such as cellular respiration but can also be formed following exposure to exogenous oxidants like air pollution, cigarette smoke or ultraviolet radiation. They have an undoubted capability to cause concomitant changes in structure and function by their action on vital cellular components including lipids, protein and nucleic acids, which are the most biologically significant targets of oxidative damage (28, 38, 39).

The body is equipped with several defence systems against ROS and RNS and the subsequent oxidative damage. These include endogenous enzymes (such as catalase, glutathione reductases and peroxidases, superoxide dismutases and paraoxonase), other endogenous proteins and proteins and molecules (e.g. glutathione, urate and coenzyme Q), repair enzymes (e.g. DNA glycosylases) and nutritional factors (e.g. vitamin E) (28, 39). The imbalanced state, in which the capacity of these in vivo antioxidant defence systems does not extend to the level of oxidative harm, is called oxidative stress.

2.1.2 Measurement of lipid peroxidation

The development of an ideal assay to measure lipid peroxidation has turned out to be a challenging task and until now there does not seem to be an agreement with regard to a reference method (40). Oxidative damage to lipoproteins, especially to LDL has been predicted to pose enhanced atherogenic effects (36). Difficulties in determining oxidized LDL are in part because LDL oxidation may involve various constituents of LDL (e.g. polyunsaturated fatty acids, cholesterol, protein), and each of these can give rise to a number of different kinds of oxidation products (41). Traditionally oxidative damage to lipids has
been assessed by an assay measuring the thiobarbituric acid reactive substances (TBARS). The test result is thought to reflect the production of malondialdehyde (MDA), a non-lipophilic peroxidation product of polyunsaturated fatty acids (PUFAs) (39). The lack of specificity of this assay has however caused concern and TBARS have been proposed to serve only as a screening test indicative of lipid peroxidation (42). MDA was also demonstrated to vary in response to oxidized lipids present in the diet, which thus makes the use of it as a marker of lipid peroxidation questionable, even when assessed more precisely by high-performance liquid chromatography (HPLC) in studies with diet changes (43).

The uncertainty in extrapolating in vitro results to humans has created the need to develop a reliable noninvasive approach to assess oxidative stress status in vivo. Currently available markers of oxidative damage occurring in vivo can be classified into three major groups: markers of oxidative damage to lipids, proteins, and DNA (39). There seems to be a growing consensus that of the biomarkers assessing lipid peroxidation in the human body, F₂-isoprostanes represent the most valuable measurement currently available (16, 40) especially as a marker of LDL oxidation. Isoprostanes are generated initially at the site of a free radical attack of esterified arachidonate in cell membranes from which they are cleaved by phospholipases (44). First three initial arachidonoyl radicals are formed which then undergo endocyclization to form four prostaglandin H₂-like bicyclic endoperoxide intermediate regioisomers. These are then reduced to four F-ring regioisomers. The generated compounds are isomeric to prostaglandin F₂α and therefore have been termed F₂-isoprostanes (45). Several lines of evidence are in favor of the reliability of this indicator of oxidative stress. F₂-isoprostanes are shown to be rather stable and specific products of free radical-induced lipid peroxidation. They are present in detectable quantities in all biological fluids, allowing the detection of even mild oxidative stress. Additionally, the levels have been shown to be increased in conditions that predispose to the development of CVD such as diabetes, cigarette smoking and hypercholesterolemia (45). Further, there is some proof that high F₂-isoprostane concentration can be modulated by endogenous antioxidants as reviewed elsewhere (16).

2.1.3 The onset of atherosclerosis

Atherosclerosis of the arterial wall is common and often a central characteristic underlying the pathogenesis of CHD. Atherosclerosis can be described as an inflammatory process by which the vascular intima becomes thickened with lipid gruel (atheroma) and connective tissue
(sclerosis) (46). CHD is caused by atherosclerosis in coronary arteries, a process characterised by cholesterol accumulation in the arterial intima, which results in narrowing of the lumen of the arteries (15). LDLs start to accumulate at the site of vascular injury initiated by circulating factors and modulated by the local anatomy and haemodynamics. The accumulation of LDL in the neonintima is a function of the circulating concentration, blood pressure, endothelial integrity, vascular relaxation and the presence of binding sites for LDL in the proteoglycans of the intimal extracellular matrix. Hypercholesterolaemia is essential for the development of atherosclerosis and CHD is rather uncommon in societies with mean serum total cholesterol concentrations lower than some 4.5 mmol/L. Also blood pressure plays a role and atherosclerosis does not develop to the venous side of the circulation (47). At the site of accumulation LDL particles become exposed to oxidising free radicals of endogenous and exogenous origin.

The oxidation of LDL can alter its receptor binding properties. Consequently, the classic LDL receptor no longer recognises it and instead the particles are being taken up by the scavenging receptors on the macrophages. In the macrophages oxidised LDL is transported out of the intima and when macrophages are filled with cholesterol, they become foam cells. Excess LDL cholesterol in the arterial intima starts to deposit also in extracellular space and together with foam cells they are aggregated to form fatty streaks, which are then developed to atheroma and further to fibrous plaque. Fibrous plaques are covered by a cap of connective tissue with embedded smooth muscle cells (15). A rupture of a fragile fibrous cap, which releases the thrombogenic lipid core, is often the reason for sudden death from myocardial infarction due to thrombosis and occlusion of the artery that obstruct the delivery of oxygen to the heart muscle (48, 49).

2.2 Plant lignans

2.2.1 Chemical nature

The lignans form a group of plant phenols, which are characterised by a dibenzylbutane skeleton (Figure 1). In plants, lignans are biosynthesised from shimic acid and are formed from the oxidative dimerization of two C9 phenylpropane precursors possessing para oxygen groups (50). Naturally they occur mostly as conjugates of hydrophilic compounds e.g. glycosidically linked to a wide variety of different carbohydrates of differing chain length (51).
They are chemically related to polymeric lignins of the plant wall and occur typically in vascular plants practically throughout the plant kingdom. Lignans are found in roots, stems, leaves, seeds and fruits (52). In grains, plant lignans are localised in the bran layer including the aleuronic layer and are thus lost in the refinement process together with the fibre fraction (53). Resins and heartwood tissue are recognised for their much higher concentration of lignans than is usual in plants. By 1987, nearly five hundred natural lignans had been listed (52) and evidently the number is constantly growing (51). When consumed, lignan glycosides are poorly absorbed from the small intestine because of their hydrophobic nature. Given that lignans are β-glycosides, they are not easily hydrolysed by mammalian enzymes, but readily by bacterial enzymes (54).

Figure 1. Plant lignans pinoresinol, lariresinol, secoisolariciresinol, and mataresinol undergo several bacteria induced conversion processes to generate enterodiol or/and enterolactone prior to absorption (55).

2.2.2 Analytical methods

Analysing plant lignans in foodstuffs is challenging due to binding to cell structures and conjugation with carbohydrates. Mazur and co-workers developed an isotope dilution gas chromatographic-mass spectrometric (GC-MS) method to assess mataresinol and secoisolariciresinol content in foods. These were at that time considered the major plant lignans in quantity. In the technique by Mazur and co-workers, stable synthesised deuterated internal standards were used for the correction of losses during the procedure and most analyses were performed at least in duplicate on (freeze-) dried material. Extended work with this reproducible and quantitative method has provided analytical data on the content of
matairesinol and secoisolariciresinol in 52 leguminous seeds (mostly beans) (56), in 12
oilseeds and nuts (57), in 6 different grains and cereals, in 8 berries, in 4 fruits and in 14
vegetables (57), and in beverages such as tea and coffee (58). In table 1 some examples are
presented per dry weight, which can give the impression that many fruits and vegetable that
contain approximately 60-90 % water are richer sources of lignans that they actually are.
Enzymes and hot acid were used for preparing the foodstuffs. Liggins and co-workers
developed this technique further by including fewer steps in preparing the sample for the
analysis than Mazur, which resulted in decreased loses of the target compounds (59).
Unfortunately, the method was not validated entirely and only few preliminary analytical
results have been published.

Table 1. Lignan contents in foods (μg/100g dry weight, method GC-MS)

<table>
<thead>
<tr>
<th>Grain &amp; Cereal</th>
<th>Secoisolariciresinol</th>
<th>Matairesinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finn Crisp bread (60)</td>
<td>28</td>
<td>41</td>
</tr>
<tr>
<td>Wheat bran (61)</td>
<td>110</td>
<td>0</td>
</tr>
<tr>
<td>Rye bran (61)</td>
<td>132</td>
<td>167</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oilseeds &amp; Nuts</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower seed (60)</td>
<td>610</td>
<td>not determined</td>
</tr>
<tr>
<td>Flaxseed (60)</td>
<td>369 900</td>
<td>1087</td>
</tr>
<tr>
<td>Sesame seed (61)</td>
<td>90</td>
<td>606</td>
</tr>
<tr>
<td>Peanut (61)</td>
<td>298</td>
<td>trace</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Berries &amp; Fruits (61)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lingonberry</td>
<td>1510</td>
<td>0</td>
</tr>
<tr>
<td>Strawberry</td>
<td>1205</td>
<td>5</td>
</tr>
<tr>
<td>Blueberry</td>
<td>835</td>
<td>0</td>
</tr>
<tr>
<td>Black currant</td>
<td>388</td>
<td>10</td>
</tr>
<tr>
<td>Apple</td>
<td>trace</td>
<td>0</td>
</tr>
<tr>
<td>Banana</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Plum</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vegetables (61)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabbage</td>
<td>33</td>
<td>trace</td>
</tr>
<tr>
<td>Broccoli</td>
<td>414</td>
<td>23</td>
</tr>
<tr>
<td>Garlic</td>
<td>380</td>
<td>trace</td>
</tr>
<tr>
<td>Onion</td>
<td>83</td>
<td>8</td>
</tr>
<tr>
<td>Carrot</td>
<td>370</td>
<td>trace</td>
</tr>
<tr>
<td>Potato</td>
<td>10</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Brewed tea (58) (mg/kg)*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Earl Grey</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Prince of Wales</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>Japan Sencha Green Tea</td>
<td>19</td>
<td>3</td>
</tr>
</tbody>
</table>

* Amount of plant lignans in brewed tea per kg of dry tea leaves
2.3 Mammalian lignans

2.3.1 Discovery, chemical nature and method of analysis

When two phenolic compounds, which had earlier been detected during GC-MS analysis of human urinary steroids, were shown to be lignans, these compounds were thought to be of ovarian origin (62). The hypothesis was based on the evidence demonstrating that their urinary excretion followed a cyclic pattern during menstrual cycle and that the compounds were barely detectable in the urine of newborn infants. It was noted already then, that the aromatic nature of these compounds, later known as enterolactone and enterodiol (50), suggests dietary origin or formation by intestinal bacteria. However, unlike all previously know natural lignans, invariably of plant origin, these two compounds carry phenolic hydroxyl groups only in the meta position of the aromatic rings. In addition, unlike plant lignans, which are optically pure, these lignans occur in the racemic form (50). Because enterodiol and enterolactone were only found in animals as opposed to plants they became referred to as mammalian lignans to distinguish them from their plant counterparts.

Soon afterwards it was shown in germ-free and ovariectomised rats, that the mammalian lignans are formed by the intestinal bacteria (63). Germ-free rats did not produce mammalian lignans, where as ovariectomy did not affect lignan metabolism. It was also demonstrated that the bacteria are necessary for the oxidation of enterodiol to enterolactone and thus the oxidation does not take place in germ-free rats. Preliminary findings in rats suggested that excretion of mammalian lignans was subject to changes in diet. These studies were then extended to men and flaxseed supplementation resulted in a major increase in the urinary excretion of enterolactone and enterodiol (64).

More proof to support intestinal microflora as the site of mammalian lignan formation was gained by means of selective antibiotic administration to men, whose urinary excretion of both lignans became undetectable or negligible after 2-3 days of antibiotic treatment (50). To locate the site of mammalian lignan formation in humans, a crossover trial in eight patients with ileostomy was carried out. Results show that the diet high in fibre (and supposedly also high in lignans) did not alter the plasma concentrations of enterodiol and enterolactone as compared with the low fibre diet. It was concluded that although the microbial population of the ileum in ileostomists is higher than what is normal for ileum, the low serum concentrations enterodiol and enterolactone measured in the patients are explained by the limited capacity of bacteria in the ileum (65).
The notion that the rate of formation of enterolactone and enterodiol is influenced by the bowel function unravels some of the mysterious early findings. Progesterone, which decreases intestinal motility, is one possible explanation of the higher excretion levels of lignans during the luteal phase of the menstrual cycle. Another explanation could be a stimulation of the activity of the intestinal microflora due to increase in biliary estrogens. Later Borriello and co-workers developed an in vitro method, which enabled the production of mammalian lignans from dietary precursors by human faecal flora (66). Several variations of the method have been widely used in later studies.

When consumed, the plant lignan glycosides undergo a sequence of metabolic changes involving hydrolysis of the sugar moiety, dehydroxylation, demethylation and further oxidation. Matairesinol glycoside is converted to enterolactone and secoisolariciresinol diglycoside is transformed to enterodiol, which is then further oxidised to enterolactone (66). Experiments in pigs have confirmed that this process of biotransformation by the intestinal microflora takes place in the caecum and colon where the microbial activity is high compared with ileum (67). Their very weak estrogenic activity has also associated mammalian lignans with phytoestrogens (68).

Adlercreutz and co-workers developed a time-resolved fluoroimmunoassay for the determination of serum enterolactone, which facilitated the measurement of larger numbers of samples (69). After the publication of the method it was found out that the used absolute amounts of the sulfatase enzyme per sample caused non-specific fluorescence and resulted in too high values of serum enterolactone concentration. Thereupon, the modified method was published soon afterwards (70).

2.3.2 Intestinal bacteria in vitro

Laboratory methods to detect and quantify mammalian lignans were available earlier than techniques to assess the amount of lignans in foods. Thus, indirect techniques to assess the amount of plant lignans in food were used by measuring the amount of mammalian lignans produced. With slight modifications to the mentioned methods of Borriello and co-workers (66), Thompson and associates (71) introduced an in vitro fermentation with human faecal microbiota, which simulates colonic fermentation. The amounts of mammalian lignans produced from 68 common plant foods were measured. The results show that oilseeds produced the highest amounts followed by dried seaweed, beans, cereal brans, whole grain
cereals, vegetables, and fruits. The results of this method are dependent on the survival of a comprehensive library of anaerobic bacteria from the gut during intestinal passing of faeces to culture in vitro. Despite of the different methodological approaches, results with the fermentation and with Mazur's techniques are mostly in agreement with regard to the rank order of most abundant sources of plant lignans.

2.3.3 Mammalian lignan precursors and metabolites

The assumption that matairesinol and secoisolariciresinol are the only plant lignans that are converted to enterodiol and enterolactone has led to equivocal results in which the consumption of plant lignans has been calculated to be smaller than what can be expected from the amount of mammalian lignans produced (72). The existence of other mammalian lignan precursors has been suspected but only recently it was shown that the plant lignans pinosylvin, lariciresinol and in lesser extent also syringaresinol, which make up the majority of the lignans in rye bran, are readily converted to enterodiol and enterolactone when incubated with human faecal inoculum. The intestinal bacteria metabolism of pinosylvin is suspected to occur via lariciresinol, which is similar to the path of biosynthesis reported for matairesinol and secoisolariciresinol (Figure 1) (55).

To date, few studies deal with the biotransformation of lignans in the mammalian organism. Upon incubation with microsomes from rat and human liver, enterodiol was shown to give rise to seven monohydroxylated metabolites and enterolactone to six aromatic and to six aliphatic monohydroxylation products (73). It has now been demonstrated that the formation of these hydroxylated metabolites of enterolactone and enterodiol takes place also in vivo in rat. However, the biological activity of the hydroxylated metabolites remains to be solved. Recent literature mentions another mammalian lignan, enterofuran that is regarded as a minor metabolite of secoisolariciresinol (55, 59). Enterofuran can be formed by dehydration of enterodiol (59). Quantitative analyses of enterofuran concentrations from biological samples have not been published.

2.3.4 Differences in lignan concentrations between and within populations

It has been thought that the rate of the formation of mammalian lignans in the gut may be influenced by the composition of gut flora, redox level in the large bowel, intestinal transit time and bowel motility (64). These factors may account for some of the high individual variability in enterolactone production from plant precursors observed in several trials.
However, the notion that all of these factors can be modified by the diet is important. Thus interestingly, among women living for years in a monastery and sharing the same foods prepared by a central kitchen, mammalian lignan excretion in urine was found to vary greatly and even more so after women were exposed to ground flaxseed (74).

Ethnic differences in urinary lignan levels were investigated cross-sectionally in 49 women, consisting of 14 white, 15 African American, 15 Latina and 5 Japanese and dietary data was obtained by food-frequency questionnaire (75). Ethnic differences in the consumption of plant foods were reported. However, white women consumed fibre-rich foods and cruciferous vegetables more often than others. Unsurprisingly thus, enterolactone excretion was found to be significantly higher in white than in African American or Latina women. The obvious limitation of the study is that no assumptions on effects of ethnicity on mammalian lignan excretion can be made without fully controlling for diet and lignan intake.

To be able to compare lignan intake, blood concentration and urinary excretion between populations, some of the larger scale studies have been collected in table 2. Unfortunately, there are only few studies available in which the number of study participants is high enough in order to be somewhat representative of the population in question. The largest study, in which lignan intake was estimated was the Dutch Prospect-EPIC cohort (European Prospective study Into Cancer and nutrition) with 17 357 participants (76). This study gave high values for Dutch women as compared to the estimates for North American women participating in the Framingham Offspring study and in the San Francisco Bay Area Study (77, 78).

Urinary excretion of lignans was similar among Australian women and in participants from Seattle area where as for Korean women somewhat lower excretion was reported (79-81). The values presented for Dutch women are given per creatinine excretion but if an average mean for creatinine (11.5 mmol/day) is used to calculate excreted enterolactone, the yield is 5.6 μmol/day. However, these slightly higher values presented for the Dutch women have been obtained by TR-FIA, which was reported to give 30 % higher results as compared to GC-MS (82). Interestingly, the biggest differences between the study populations were observed in serum levels of enterolactone, which were highest in Finnish women free of cancer history (26 nmol/L) (83). The concentration was also high (20.2 nmol/L) in U.S. women from New York who were free of cancer or cardiovascular disease (84). In two other studies Finnish men also ranked quite high with mean plasma concentrations of 17.3 nmol/L (85) and 15.6 nmol/L (86), whereas the mean for Norwegian men was only 6.7 nmol/L (86).
2.3.5 Supplementation studies on dietary lignans

The amount of mammalian lignans excreted in urine has often been used in estimating the plant lignan content of different foods. By changing the diet given to rats and monitoring the excretion of mammalian lignans in urine, several crop plants were tested for their plant lignan content (64). These first experiments revealed the extraordinary lignan concentration of flaxseed (also known as linseed), which is still the most abundant source of lignans known to exist. Because of this obvious reason flaxseed has been used as the source of lignans in several clinical trials. Shultz and co-workers conducted a 6-week supplementation study in six men who consumed 13.5 g/day of flaxseed and observed following the intervention 7-28 fold concentrations of enterolactone in urine compared to baseline values (87). A longer study was carried out in 18 women, who consumed 10 g/day of flaxseed powder above their usual diet for three menstrual cycles. The urinary enterolactone excretion became 3- to 285-fold (88) and faecal excretion of enterolactone (in the 13 women whose sample was available) became 16-fold in response to flaxseed supplementation (89).

Morton and co-workers carried out the first supplementation studies in which the levels and changes in plasma levels of enterolactone and enterodiol were studied in response to flaxseed supplementation (90). In a 6-week experiment in 29 postmenopausal women, diets were supplemented with soy, clover sprouts or flaxseed, each for 2-week periods. During the flaxseed period plasma levels of enterolactone and enterodiol combined reached the concentration of 500 ng/ml (corresponding approximately 1667 nmol/L), where as during the other experiment periods plasma concentration of lignans was reverted to basal level. The only flaxseed supplementation trial in men, was carried out in four individuals during one day. Plasma level of enterolactone was increased at 8.5 hours after consuming the cakes containing 15 g of cracked flaxseed but the maximal concentration was reached after 24 hours, which were on average eight times higher than the baseline values.

Nebbitt and co-workers conducted an experiment, where nine women supplemented their diets with 5, 15, 25 g of raw or 25 g processed flaxseed for seven days (91). This study showed a dose-dependent urinary lignan response to flaxseed but the plateau level for plasma enterolactone could not be established. Also in postmenopausal women flaxseed consumption affected enterolactone excretion in a dose-dependent manner (74). This randomised, crossover trial consisted of three seven-week study periods, during which the 31 participants recruited from the local monastery supplemented their diet with 0, 5 or 10 g of ground flaxseed/day.
Studies using experimental diets of common foodstuffs, other than flaxseeds to study their effect on lignan excretion have been less common. It was shown in 11 men and in 9 women who all consumed four 9-day experimental diets, that urinary excretion of enterolactone and enterodiol was significantly higher during the carotenoid (carrot and spinach) and cruciferous (broccoli and cauliflower) vegetable diets than during a vegetable-free diet (92). With a similar study design it was shown, that during a vegetable and fruit diet the excretion of enterodiol was increased compared with the basal and with legume and allium diets, but the diets did not affect enterolactone excretion (93). However, compared with the results from flaxseed feeding trials, the changes in lignan excretion observed in these studies are small. In Finnish participants, 219-162 g of rye bread was not able to increase the serum level of enterolactone from the baseline level. But during the 4-week period of wheat bread only, enterolactone concentration was halved from baseline values (72). These participants were however living in the Kuopio area, where rye bread is commonly consumed in high amounts. In another Finnish study, where the consumption of vegetables, fruits and berries was increased in a 12-week dietary intervention, serum enterolactone concentration increased as well (70). This dietary intervention study, with 85 middle-aged participants, was aimed at hypertensive individuals and their spouses and thus provided useful information of the feasibility and of the effects of a population intervention in small scale.

2.3.6 Dietary determinants of lignan concentrations in observational studies

Cross-sectional associations between diet and mammalian lignans have been investigated in several studies. In 26 women from Boston who consumed their habitual diets a positive correlation ($r=0.59$) between the intake of fibre and urinary enterolactone was reported (94). In another study the intake of grain products was shown to correlate amazingly strongly ($r=0.996$) with urinary enterolactone in 53 women from Boston and Helsinki (95). Later these associations have been confirmed in larger population samples but the correlations have been modest compared to the previous. From a study of 98 participants it was reported that urinary lignan (enterolactone and enterodiol) excretion was 50 % higher ($P=0.04$) among the participants with high vegetable and fruit intake ($\geq5$ servings/dny), than among the participants who consumed two or less servings per day (80). In 2380 Finnish participants serum enterolactone concentration had a positive association with the consumption of whole-grain products and fruit and berries in men (85). Among women, an association was seen with serum enterolactone concentration and the intake of vegetables. This data set was expanded to
2753 Finnish men and women and the impact of oral antimicrobials on serum enterolactone concentration was examined. The results show that the use of oral antimicrobials up to 12-16 months before serum sampling had a significant lowering effect on serum enterolactone concentration (96).

Another recent cross-sectional study on 193 healthy young men and women from Seattle examined associations between dietary intake and plasma enterolactone concentration (97). The strongest correlation existed between total fibre and plasma enterolactone concentration (r=0.36) and in a multivariate model dietary fibre intake accounted for 13% of the variability of plasma enterolactone concentration. The addition of alcohol and caffeine along with demographic characteristics to the model interestingly increased this ratio and together they explained up to 22% of the variability. Participants who consumed 0.5-1 alcohol drinks/day had 131% higher plasma enterolactone concentrations than non-drinkers. Importantly though, one of the exclusion criteria was alcohol consumption over two drinks per day thus heavy consumers were not included. Consequently, the observed differences in plasma enterolactone concentration between non-drinkers and moderate alcohol consumers may be due to the distinct health behaviour pattern of abstainers.
Table 2. Lignan intake, blood concentration and urinary excretion in various study populations.

<table>
<thead>
<tr>
<th>Name/ description of the study</th>
<th>Study population</th>
<th>Age (y)</th>
<th>Measurement method</th>
<th>Medium</th>
<th>Median/ Mean values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutch Prospect-EPIC cohort (76)</td>
<td>17 357 F</td>
<td>50-69</td>
<td>FFQ</td>
<td>Diet</td>
<td>Mat 70 µg/d, Seco 930 µg/d</td>
</tr>
<tr>
<td>Framingham Offspring Study (77)</td>
<td>964 F</td>
<td>-</td>
<td>FFQ</td>
<td>Diet</td>
<td>Mat 19 µg/d, Seco 560 µg/d</td>
</tr>
<tr>
<td>San Francisco Bay Area Study (78)</td>
<td>1610 F</td>
<td>35-79</td>
<td>FFQ</td>
<td>Diet</td>
<td>Mat ~30 µg/d, Seco ~100 µg/d</td>
</tr>
<tr>
<td>‘Australian Case-Control Breast Cancer Study’ (79)</td>
<td>144 F</td>
<td>30-84</td>
<td>ID-GC-MS</td>
<td>Urine (72-h)</td>
<td>Enl 3.1 µmol/d</td>
</tr>
<tr>
<td>‘Seattle Area Dietary Determinants of Plasma Enterolactone’ (80)</td>
<td>98 F + M</td>
<td>18-37</td>
<td>ID-GC-MS</td>
<td>Urine (72-h)</td>
<td>Enl 3.6 µmol/d, Ediol 1.3 µmol/d</td>
</tr>
<tr>
<td>‘Bone Mineral Density in Korea’ (81)</td>
<td>75 F</td>
<td>52-65</td>
<td>GC-MS</td>
<td>Urine (24-h)</td>
<td>Enl 1.5 µmol/d, Ediol 0.4 µmol/d</td>
</tr>
<tr>
<td>‘Dutch breast cancer screening program’ (82)</td>
<td>268 F</td>
<td>50-64</td>
<td>TR-FIA</td>
<td>Urine (2 x morning sample)</td>
<td>Enl/creatinine 483.9 µmol/mol (Enl 5.6 µmol/d)</td>
</tr>
<tr>
<td>‘Nordic cancer registry linkage’ (86)</td>
<td>3344 M</td>
<td>40-60</td>
<td>TR-FIA</td>
<td>Serum</td>
<td>Enl 8.4 nmol/L (cohort mean), 15.67 nmol/L (Fin), 6.7 nmol/L (Nor)</td>
</tr>
<tr>
<td>‘Finnish cross-sectional survey’ (85)</td>
<td>2380 M + F</td>
<td>25-64</td>
<td>TR-FIA</td>
<td>Serum</td>
<td>Enl 17.3 nmol/L (in men), 20.5 nmol/L (in women)</td>
</tr>
<tr>
<td>New York University Women’s Health Study (84)</td>
<td>60 F</td>
<td>34-65</td>
<td>TR-FIA</td>
<td>Serum</td>
<td>Enl 20.2 nmol/L, Ediol 1.5 nmol/L</td>
</tr>
<tr>
<td>Kuopio Breast Cancer Study (83)</td>
<td>208 F</td>
<td>25-75</td>
<td>TR-FIA</td>
<td>Serum</td>
<td>Enl 26 nmol/L</td>
</tr>
</tbody>
</table>

F = female, M = male, y = year, CVD = cardiovascular disease, FFQ = food frequency questionnaire, (ID-)GC-MS = (isotope dilution) gas chromatography-mass spectrometry, h = hour, d = day, Enl = enterolactone, Ediol = enterodiol, Mat = Mataresinol, Seco = secoisolariciresinol
* Values are rough estimates based on the distribution presented by the authors.
† Assuming the average creatinine excretion is 11.5 nmol/d.
‡ Values are estimates of means calculated from values given separately for cases and controls.
2.4 Health effects of lignans

2.4.1 Experimental evidence

Polyphenols are often considered to contribute to the pool of antioxidants in plant foods. Cumulative experimental evidence from in vitro assays support this assumption and some recently conducted studies on humans are also in favour of polyphenols’ antioxidant activity but the results remain inconsistent (34, 37, 98). While most published work on humans has been concentrated on the antioxidant effects of flavonoids, the enormous heterogeneity even among this group of compounds limits the plausibility of extrapolating these results e.g. to lignans. The term polyphenols refers to substances that comprise an aromatic ring with one or more hydroxyl constituents (37). They are products of secondary metabolism of plants and form an extremely wide and complex group of compounds (99). The antioxidant potential is thought to be mediated through the number of phenolic hydroxyl groups attached to ring structures which enable polyphenols to act as reducing agents, hydrogen donating antioxidants, and singlet oxygen quenchers (34, 98). These properties as well as absorption and metabolism are dependent on factors such as degree of glycosylation/acylation, conjugation with other phenolics, molecular size and degree of polymerisation (98). As a whole, a better understanding of the bioavailability of polyphenols is critical before an extensive evaluation of their biological value is feasible.

Studies aimed to test the antioxidant effects specifically of lignans are practically nonexistent but there are many papers, in which the antioxidant capacity has been tested by supplementing study participants with specific foodstuffs known to be rich sources of a polyphenol of interest and concurrently containing lignans. Red wine and tea, which are abundant sources of phenolic acids and of the flavonoid catechin, have been common supplements in human trials but also fruits and vegetables have been used. Undoubtedly, the average concentrations of lignans in black tea (~19 mg/L) (100) and in red wine (~0.8 mg/L) (100) are small compared to those of phenolic acids in red wine (200 mg/L) (37) and flavonoids in tea (~21-54 mg/L) (101). Nonetheless, the low content does not necessarily preclude lignans from participating in the defence against oxidative stress. Morton and co-workers have reviewed human trials on the antioxidant effects of wine, fruit juice and tea but the results appear largely inconclusive, partly due to differences in study design and methodology of LDL isolation and oxidation conditions (37). In more recent studies similar inconsistencies have been reported as well. Non-alcoholic red wine has been used as the
source of polyphenols in few studies. Neither a single dose (which equals to five red wine equivalents mL/kg) of red wine nor dealcoholised red wine had any effect on ex vivo serum or LDL oxidation (102) but a non-alcoholic red wine extract (equivalent to 375 mL of red wine) supplementation for two weeks inhibited copper-initiated oxidation of LDL in another study (103). Dealcoholized red wine, consumed for 500 mL each evening for two weeks was shown to decrease plasma F2-isoprostanes significantly in men (104). The results from recent studies on black tea have not been as promising. McAnlis and co-workers concluded from a small cross-over trial that consumption of moderate quantities of black tea does not increase plasma total antioxidant capacity or alter the susceptibility of LDL to oxidation acutely or after one week of consumption. In continuum, the 2-week supplementation of black tea (300 mL) and onions (150 g) had no significant effect on plasma F2-isoprostane concentrations or on MDA-LDL autoantibody titer (105). Similarly disappointing results were reported from a 3-week placebo-controlled cross-over trial in male smokers, in which a daily vegetable burger (~100 g, including 500 g of mixed vegetables) or a fruit drink (~330 mL) demonstrated no effects on markers of oxidative damage to lipids (including F2-isoprostanes), proteins or DNA (106). These results exemplify some of the hardship in trying to show the impact of dietary polyphenols on oxidative stress. Thus, it is preliminary to make assumptions of the role of polyphenols as antioxidants in disease prevention, as even the causal relation between oxidative stress and chronic diseases still needs to be confirmed.

To address other published effects of lignans, the evidence is mostly limited to in vitro and animal experiments. Secoisolariciresinol diglycoside, isolated from defatted flaxseed, was suggested to have hydroxyl radical-scavenging properties (107). Additionally secoisolariciresinol, enterolactone and enterodiol were reported to act as antioxidants in lipid and aqueous in vitro model systems (108). Flaxseed, with high amounts of α-linolenic acid and secoisolariciresinol, was reported to lower serum cholesterol and decrease thrombin-stimulated platelet aggregation in a 3-month feeding trial in humans (109). In rabbits flaxseed was shown to reduce atherosclerosis (110, 111). The anticancer effect of flaxseed and secoisolariciresinol diglycoside was investigated in rats and the authors concluded that the observed protective effect may be related, at least partly, to reduction in plasma insulin-like growth factor I (IGF-I) which is associated with increased breast cancer risk (112). In similar studies in rats that have associated lignans with cancer protection, flaxseed and secoisolariciresinol have been shown to increase cecal β-glucuronidase activity (113). Secoisolariciresinol was also reported to be antiestrogenic without gross tissue toxicity (114) and beneficial throughout the promotional phase of carcinogenesis (115, 116).
2.4.2 Epidemiological evidence concerning disease risk

In previous published population studies, lignans have been associated with cancer risk (Table 3). Several case-control breast cancer studies have been published, where urinary enterolactone concentration has been measured. In an Australian study, in which 144 pairs were included in the analysis, the trend across urinary enterolactone quartiles remained significant (P=0.013) even after adjustment for other risk factors and there was a risk reduction of 64% among women in the highest quarter (79). The results of a Finnish study, with 194 breast cancer cases and 208 controls, were consistent with this finding (83) but another Dutch study of 88 breast cancer cases and 268 controls presented conflicting results (82). In the mentioned Dutch study, higher excretion (enterolactone/creatinine ratio) measured from spot urine samples was weakly and non-significantly associated with an increased breast cancer risk. The median level of exposure was in the same order of magnitude as in the Australian study but the study consisted of exclusively of postmenopausal women. Additionally, urinary excretion of enterolactone measured in spot urine samples might not be comparable to values measured in pools of 72-hour urinary excretion. Dietary lignan intake was also shown to have a non-significant association with increased breast cancer risk in both pre- and postmenopausal San Franciscan women (78). The levels of lignan consumption were considerable low but interestingly, in a different sample from Bay Area Cancer study, the women in the highest fifth (≥161 μg/day) of lignan consumption were at decreased thyroid cancer risk (117). Unfortunately, the most relevant study concerning this thesis is the Dutch study showing lignan intake to have a strong protective effect against cardiovascular events, is until now only available as a meeting abstract (118). Thus thorough analysis of its results cannot be made yet.
Table 3. Lignans and disease risk.

<table>
<thead>
<tr>
<th>Name/description of the study</th>
<th>Measurement</th>
<th>Outcome</th>
<th>Incident cases vs. controls/cohorts</th>
<th>Duration of follow-up (y)</th>
<th>Age</th>
<th>Sex</th>
<th>Main result (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutch Prospect-EPIC cohort (118)</td>
<td>Lignan intake (FFQ)</td>
<td>Cardiovascular event</td>
<td>829 vs. 16 111</td>
<td>4.3</td>
<td>49-70</td>
<td>F</td>
<td>RR 0.72 (0.57-0.91) for highest vs. lowest quarter</td>
</tr>
<tr>
<td>‘Australian Case-Control Breast Cancer Study’ (79)</td>
<td>E1n (72-h urine)</td>
<td>Breast cancer</td>
<td>144 vs. 144</td>
<td>-</td>
<td>30-84</td>
<td>F</td>
<td>OR 0.36 (0.15-0.86) for highest vs. lowest quarter</td>
</tr>
<tr>
<td>‘Dutch Breast Cancer Screening Program’ (82)</td>
<td>E1n/Creatinine (2 x urine sample)</td>
<td>Breast cancer</td>
<td>88 vs. 268</td>
<td>1.9</td>
<td>50-64</td>
<td>F</td>
<td>OR 1.43 (0.79-2.56) for highest vs. lowest third</td>
</tr>
<tr>
<td>Kuopio Breast Cancer Study (83)</td>
<td>E1n (serum)</td>
<td>Breast cancer</td>
<td>194 vs. 208</td>
<td>-</td>
<td>25-75</td>
<td>F</td>
<td>OR 0.38 (0.18-0.77) for highest vs. lowest fifth</td>
</tr>
<tr>
<td>Bay Area Breast Cancer Study (78)</td>
<td>Mat and Seco intake (FFQ)</td>
<td>Breast cancer</td>
<td>1 326 vs. 1 657</td>
<td>-</td>
<td>35-79</td>
<td>F</td>
<td>OR 1.3 (1.0-1.6) for highest vs. lowest quarter</td>
</tr>
<tr>
<td>‘Nordic Cancer Registry Linkage Study’ (86)</td>
<td>E1n (serum)</td>
<td>Prostate cancer</td>
<td>794 vs. 2 550</td>
<td>14.2</td>
<td>40-60</td>
<td>M</td>
<td>OR 1.08 (0.83-1.39) for highest vs. lowest quarter</td>
</tr>
<tr>
<td>San Francisco Bay Area Study 2001 (117)</td>
<td>Seco intake (FFQ)</td>
<td>Thyroid cancer</td>
<td>608 vs. 558</td>
<td>-</td>
<td>35-79</td>
<td>F</td>
<td>OR 0.56 (0.35-0.89) for highest vs. lowest fifth</td>
</tr>
<tr>
<td>‘Bone Mineral Density in Korea’ (81)</td>
<td>E1n (24-h urine)</td>
<td>Degree of bone mineral density</td>
<td>75</td>
<td>-</td>
<td>52-65</td>
<td>F</td>
<td>Correlation of 0.39, P&lt;0.01 between bone mineral density and urinary excretion of enterolactone</td>
</tr>
</tbody>
</table>

E1n = enterolactone, Mat = matairesinol, Seco = secoisolariciresinol
FFQ = food frequency questionnaire, h = hour, y = year, CI = confidence interval, F = female, M = male, RR = relative risk, OR = odds ratio
3 AIMS OF THE STUDY

The purpose of these studies was to investigate the role of serum enterolactone concentration in predicting CVD risk and its association with CVD risk factors. The aim was also to carry out a phloem bread supplementation trial and to assess the consequent changes in serum enterolactone concentration.

The specific aims of the study were to:

**Original study I**
Investigate the relationship between serum enterolactone level and the risk of acute coronary events in men.

**Original study II**
Investigate the role of serum enterolactone level in predicting the risk of CHD-related, CVD-related and all-cause mortality in a long-term follow-up.

**Original study III**
Study the association between serum enterolactone concentration and lipid peroxidation, assessed by plasma F$_{2}$-isoprostanes.

**Original study IV**
To study the sensitivity of serum enterolactone concentration in detecting changes in dietary lignans, and to investigate the possibility of using phloem supplementation in rye bread to increase serum enterolactone level.
4 MATERIAL AND METHODS

4.1 Study populations

4.1.1 Kuopio Ischaemic Heart Disease Risk Factor Study

The KIHD study is an on-going population-based study of risk factors for CVD, atherosclerosis, and related outcomes in men from the Kuopio region in eastern Finland (119). The study protocol was approved by the Research Ethics Committee of the University of Kuopio. The study population is a random sample of men living in the city of Kuopio or neighbouring rural communities. The study sample was composed of 3235 men, stratified and balanced in four strata: aged 42, 48, 54, or 60 years at baseline examination. Of these, 2682 (83 %) participated. The baseline examinations were carried out between March 1984 and December 1989.

4.1.1.1 Examination protocol

The KIHD baseline examinations were carried out over two days, one week apart and consisted of a wide variety of biochemical, physiological, anthropometric, and psychosocial measures, as described earlier in detail (119). At the first visit a nurse measured body height and weight, waist and hip circumference and blood pressure and a medical examination and a maximal, symptom limited cycle exercise test was performed. Participants were instructed to complete a four-day food record and to collect 24-hour urine samples, which would be brought to the research institute on the second visit.

For the second visit participants were instructed to fast over night and to abstain from smoking for 12 hours, from using alcohol for 3 days or analgesics for 7 days. On the visit participants gave blood specimens for laboratory determinations between 8 and 10 a.m. after resting in supine position for 30 minutes. Blood samples were obtained by venipuncture and collected into vacuum tubes (Venoject; Terumo, Leuven, Belgium). The 4-day food record completed at home was checked together with a nutritionist.

4.1.1.2 Ascertainment of follow-up events

The province of Kuopio participated in the multinational MONICA (Monitoring of Trends andDeterminants of Cardiovascular Disease) project (120), in which detailed diagnostic information of all coronary events that occurred by December 1992 was collected
prospectively. The diagnostic classification was made by the FINMONICA coronary registry group (119). In the original study I, data on acute coronary events between January 1993 and December 1996 were obtained by computer linkage to the national hospital discharge register and classified by a physician using identical diagnostic criteria, including symptoms, cardiac enzymes and electrocardiographic findings as explained previously (120). If multiple non-fatal events occurred during the follow-up, the first event for each participant was considered as end point for the analyses.

In the original study II, deaths were ascertained by record linkage to the national death registry using the Finnish social security number. There were no losses to follow-up. All deaths that occurred during study enrolment (from March 20, 1984, to December 5, 1989) and December 31, 1999, were included. Deaths that were coded with the *International Classification of Diseases, Ninth Revision* (ICD-9)(1977), codes 390 to 459 were included in the analyses of CVD-related deaths.

### 4.1.1.3 Exclusion of participants

Medical history, use of medications and family history of diseases were assessed using a self-administered questionnaire. Prevalent CHD was defined as either a history of acute coronary event or angina pectoris or positive angina pectoris on effort in Rose interview or the use of nitroglycerin tablets once a week or more frequently. In the original study I men with prevalent CHD at baseline (n=677) were excluded from the analyses, as the presence of clinical CHD could have influenced dietary habits. In the present study the cohort was followed-up on average 10 years, by the end of 1996, during which 81 definite and 55 probable acute coronary events and 31 typical long episodes of chest pain occurred. To ensure the comparability of the controls, one control from the same cohort was matched for each case according to age (42, 48, 54, 60), examination year (1984-1989) and place of residence (the same municipality out of ten). Thus, the study included a total of 334 participants.

In the original study II baseline serum samples for the enterolactone analysis were only available for 2557 participants, hence others were excluded. One participant was withdrawn due to exceptionally high enterolactone concentration (205.1 nmol/L) considered as a clear outlier. Men with prevalent CHD at baseline (n=638) or history of stroke (n=29) were excluded, thus the study sample included 1889 men free of CVD with this restricted definition criteria of CVD. During the follow-up time of 12.2 years we documented 70 CHD-related, 103 CVD-related and 242 all-cause deaths.
4.1.2 Antioxidant Supplementation in Atherosclerosis Prevention Study

The ASAP Study is a 2x2 factorial placebo-controlled randomised trial to study the effects of a special formulation of vitamin E and slow-released vitamin C supplementation on oxidative stress, lipid peroxidation, atherosclerotic progression, and the incidence of upper respiratory infections in high risk men and women (40). The study protocol was approved by the Research Ethics Committee of the University of Kuopio.

4.1.2.1 Recruitment of participants

The participants were regularly smoking (≥5 cigarettes/day) or non-smoking men and postmenopausal women aged 45-69 years with a serum cholesterol concentration 5.0 mmol/L or over at a screening visit. The exclusions included e.g. uncontrolled hypertension and severe diseases. A total of 520 participants (256 men, 264 women) were randomised into the trial. For the baseline visit participants were instructed to fast for 12 h and to abstain from alcohol use for a week before blood sampling. Between 8 and 10 a.m., after the participant had rested in sitting position for 5 min, blood was drawn into Venoject vacuum tubes (Terumo Corp., Belgium). On the day before blood drawing, a 24-hour urine was collected. Intensity of conditioning activity, smoking and mean weekly alcohol consumption (based on 12-month use) were assessed by a separate questionnaires. The consumption of foods was assessed by a 4-day food record checked by a nutritionist. Baseline visits were carried out between October 1994 and October 1995.

4.1.2.2 Study sample

The original study III is based on a subsample of 100 consecutive male participants for whom plasma F₂-isoprostane and serum enterolactone were assayed from samples taken at baseline. Of these men, a half smoked more than one cigarette per day.

4.1.3 Phloem fortification study

Phloem supplementation study is a placebo-controlled randomised trial to study the effects of phloem powder forticated rye bread on serum polyphenol concentrations. Phloem powder is a concentrated source of plant lignans as well as catechins.
4.1.3.1 Recruitment of participants

Seventy-five non-smoking voluntary men aged 30-69 years were recruited from the Kuopio area in eastern Finland through newspaper advertisements. Potential participants were screened in an initial telephone interview for the following inclusion criteria: 1) No severe obesity (body mass index (BMI) <32 kg/m²), 2) increased serum cholesterol concentration (total cholesterol 6-9 mmol/L), 3) no regular use of any drug with antioxidative (like supplements of selenium, vitamins C and E and β-carotene) or lipid lowering properties 4) no chronic diseases such as diabetes, CHD or other major illness and 5) willingness to consume 70 g of dried rye bread/day for four weeks. All criteria were ascertained prior to entering the study and participants were also examined by a physician. A written informed consent approved by the local Ethics Committee was obtained from all participants. The study protocol was approved by the Research Ethics Committee of the University of Kuopio.

4.1.3.2 Study design

The study was a 4-week randomised double-blind supplementation study. Participants were randomly assigned to one of the three study groups, each consuming different study breads: a rye bread high in phloem powder (HP group), a rye bread low in phloem powder (LP group) or placebo rye bread (placebo group). In the bread consumed by the LP and HP groups, 7% and 14% respectively of the rye flour used in baking was substituted with phloem powder (Table 4). Due to logistics, the study was conducted in two parts. First, 15 volunteers were randomised both into the placebo group and to the LP group. The second part was started immediately after the first part. In the second phase of the study we recruited and randomised 15 volunteers more to the placebo and LP groups and added the HC group to the study with 15 volunteers, the total amount of participants then summing up to 75. The daily amount of study bread was 70 g, which was instructed to be consumed evenly throughout the day.

4.1.3.3 Examination protocol

Four weeks prior to the study participants were asked to discontinue tea and red wine drinking and the consumption of chocolate. Other than these, participants were advised to maintain their dietary and other lifestyle habits such as exercise unchanged. It was emphasised to the study participants not to cut down on their normal bread consumption. A 4-day food record (with one weekend day included) was required during the week before the intervention and during the last week of intervention period to control for possible confounding in the diet and
compliance to given instructions and the records were checked by a nutritionist at the visit. Fasting blood samples were collected at baseline and after the 4-week intervention period together with blood pressure and weight measurements. Determination of serum enterolactone concentration and the other measurements were carried out after the conclusion of both of the study phases.

Table 4. Lignan content of the rye bread and the phloem rye bread.

<table>
<thead>
<tr>
<th>nmol / 70 g¹</th>
<th>Placebo (PB)</th>
<th>Low phloem (LP, 7 %)</th>
<th>High phloem (HP, 14 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secoisolariciresinol</td>
<td>79</td>
<td>11493</td>
<td>19729</td>
</tr>
<tr>
<td>Matairesinol</td>
<td>60</td>
<td>61</td>
<td>164</td>
</tr>
<tr>
<td>Larioresinol</td>
<td>93</td>
<td>148</td>
<td>180</td>
</tr>
<tr>
<td>Pinoresinol</td>
<td>93</td>
<td>120</td>
<td>140</td>
</tr>
<tr>
<td>Syringaresinol</td>
<td>433</td>
<td>509</td>
<td>560</td>
</tr>
<tr>
<td>Isolariciresinol</td>
<td>240</td>
<td>610</td>
<td>865</td>
</tr>
<tr>
<td><strong>Total lignans</strong></td>
<td><strong>998</strong></td>
<td><strong>12941</strong></td>
<td><strong>21638</strong></td>
</tr>
<tr>
<td><strong>Total precursors</strong>²</td>
<td><strong>758</strong></td>
<td><strong>12331</strong></td>
<td><strong>20773</strong></td>
</tr>
</tbody>
</table>

¹ Values are given nmol/70 g, which was the amount of daily bread supplementation. ² Isolariciresinol is not included

4.1.3.4 Study sample

All 75 men recruited for the study completed the supplementation period and provided the necessary blood samples. Two participants were excluded, one due to pathological concentrations of serum triglycerides (8.8 mmol/L) and the other due to exceptionally high level of serum enterolactone (337 nmol/L), leaving 73 men for the final analysis. Thus the final analysis included 29 men in the placebo group, 29 men in the LP group and 15 men in the HP group.
4.2 Biochemical analyses

4.2.1 Measurement of serum enterolactone concentration

Determination of serum enterolactone concentration was based on time-resolved fluorometric immunoassay (TR-FIA) using a europium chelate as a label, described previously (69, 121). After synthesis of 5-O-carboxymethylenterolactone the compound was coupled to bovine serum albumin and then used as antigen for immunising the rabbits. The tracer with the europium chelate was synthesized using the same 5-derivate of enterolactone. After enzymatic hydrolysis and ether extraction the immunoassay was carried out using the VICTOR 1420 multilabel counter (Wallac, Turku, Finland). No antiserum cross-reactivity with available lignans, isoflavonoids or flavonoids could be detected. The working range of the assay was 1.5–540 nmol/L. The intra-assay and interassay coefficients of variation at three different concentrations varied from 4.5 to 9.2 % and from 5.9 to 9.1 %, respectively. The correlation coefficient for 44 samples between this method and our reference isotope dilution GC-MS method was 0.82 (P=0.004), the mean values being 31.2 nmol/L for the GC-MS assay and 31.0 nmol/L for the TR-FIA assay. The analyses were carried out at the Folkhälsan Research Center, Biomedicum, Helsinki, Finland.

4.2.2 Measurement of plasma F₂-isoprostane concentration

F₂-Isoprostane concentrations in EDTA-plasma samples taken in the ASAP study at baseline were determined at Vanderbilt University Medical Center, Nashville, USA, in 1997. A deuterated prostaglandin F₂α internal standard was added to plasma and F₂-isoprostanes were extracted with C₁₈ and silica minicolumns. Compounds were converted to pentafluorobenzyl ester trimethylsilyl ether derivatives and analysed by a GC-MS assay (122). The coefficient of variation for both intraday and interday variation was 9 %.

4.2.3 Other laboratory measurements

The cholesterol contents of serum lipoprotein fractions and triglycerides were determined enzymatically (Boehringer Mannheim, Mannheim, Germany and Konelab, Espoo, Finland) (123). Serum apolipoprotein B was determined by an immunoturbidimetric method (Konelab, Espoo, Finland). High density lipoprotein (HDL) and its subfractions were separated from fresh serum samples using ultracentrifugation and precipitation (123). HDL was measured
from supernatant after magnesium chloride-dextran sulphate precipitation. Plasma fibrinogen determination was based on clotting of diluted plasma with excess thrombin using a Coagulometer KC4 device (Heinrich Amelung, Lemgo, Germany). Serum ferritin was measured by radioimmunoassay (Amersham International, Amersham, U.K.). Blood glucose was measured using the glucose dehydrogenase method (Merck, Darmstadt, Germany). Diabetes was defined as fasting blood glucose of ≥26.7 mmol/L or a clinical diagnosis of diabetes with either dietary, oral or insulin treatment. The 24-hour urinary excretion of nicotine metabolites was measured by a colorimetric method (Erilab, Kuopio, Finland) (124).

Plasma ascorbic acid concentration was determined by HPLC. In the ASAP study plasma α-tocopherol and β-carotene were determined by HPLC at the Research Institute of Public Health, Kuopio, Finland as described earlier (125). To separate the effect of α-tocopherol from that of serum lipids, values for lipid standardized α-tocopherol was used in the statistical analysis (126). Plasma total homocysteine (tHcy) concentrations were measured at the National Public Health Institute, Helsinki, Finland, essentially as described by Araki and Sako (127). Homocysteine, other mixed disulfides and the protein bound homocysteine were first reduced to free homocysteine by tributylphosphine and tHcy was measured using an isocratic reversed-phase HPLC method with fluorescence detection as described earlier in detail (128).

4.3 Assessment of dietary intake of nutrients

Dietary intake of nutrients and energy was assessed by using four day food recording. Instructions were given and completed food records were checked by a nutritionist. Intake of nutrients was estimated using the NUTRIC® software. The data bank of NUTRIC® is compiled using mainly Finnish values of nutrient composition of foods. Food records were calculated for the original study I in the beginning of 1990 using NUTRIC® version 1.0 and for the original studies II-IV in 2000 using NUTRIC® version 2.5, which takes into account losses of vitamins in food preparation. For the original study II all nutrients were adjusted for dietary energy intake using the residual method (129). Energy adjustment is based on the notion that a larger, more physically active person requires a higher caloric intake, which is associated with a higher absolute intake of all nutrients. Therefore energy adjustment takes into account differences in energy requirements among individuals. The residuals were standardized by the mean nutrient intake of a participant consuming 10 MJ/day, the approximate average total energy intake in this study population.
4.4 Lignan analysis of phloem powder and of the study breads

Lignan content of phloem powder and of the study breads was determined applying the GC-MS method previously described (60). The method used was developed to analyse secoisolariciresinol and matairesinol from food samples and it was not separately optimised for the analysis of the newly discovered rye lignans; lariciresinol, pinosylvin, syringaresinol and isolariciresinol. Acid hydrolysis included in the method apparently partly destroyed other new rye lignans but did not affect the amount of isolariciresinol. Therefore the presented results concerning the newly discovered lignans are semi-quantitative, the true values being higher.

4.5 Other measurements

In the KIHD study two trained nurses measured resting blood pressure by with a random-zero mercury sphygmomanometer (Hawksley, United Kingdom). In the KIHD study the measuring protocol included, after supine rest of five minutes, three measurements in supine, one in standing and two in sitting position with five minutes’ intervals. The mean of all six measurements was used as the systolic and diastolic blood pressure. Participants with elevated systolic (≥160 mm Hg) or diastolic (≥95 mm Hg) blood pressure or who used antihypertensive drugs were classified as hypertensive. In the ASAP study resting blood pressure was measured three times in the sitting position at five minutes’ intervals, and the mean was used. In the phloem trial, blood pressure was measured manually in sitting position after a rest of 10 min, three measurements at 3-min intervals.

In the KIHD study assessments of ischemic findings in exercise test (130), smoking (123) medical history and medications (123), and family history of disease (123) were carried out as described previously. Family history of CHD was defined as positive if either biologic parents or siblings had CHD. Frequency of constipation and bronchitis and other infections was assessed by a questionnaire. Maximal oxygen uptake as a measure of cardiorespiratory fitness was assessed at KIHD study baseline with a maximal symptom-limited exercise test on an electrically-braked cycle ergometer, as explained in detail elsewhere (130). Body weight was measured by a balance scale in the KIHD study and by an electronic scale in ASAP and
phlebem studies. Body mass index (BMI) was computed by dividing body weight in kilograms by the square of height in meters.

The current number of cigarettes, cigars, and pipe-fulls of tobacco smoked daily and the duration of regular smoking were recorded using a self-administrated questionnaire. The lifelong exposure to smoking (cigarette years) was estimated as the product of years smoked and the number of cigarettes or other smoked daily at the time of examination. Cigarette pack-years was computed by dividing cigarette years by 20 (the number of cigarettes in a pack). A self-reported quantity-frequency questionnaire was used to record the level of alcohol use. The average weekly consumption of alcohol in pure ethanol (g/week) was calculated based on the known alcohol content of each beverage type and the reported doses and frequencies of drinking sessions, described previously (131).

4.6 Statistical analysis

In the original study I the differences in risk factors between the cases and controls were tested for statistical significance with Student's t-test allowing for unequal variances and those between serum enterolactone quarters with one-way variance analysis. Risk-factor-adjusted odd ratios (OR) for AMI were estimated by forced and stepwise conditional multivariate logistic regression models. Confidence intervals (CI) were estimated based on the assumption of asymptotic normality of estimates. Missing values in covariates were replaced by means, separately for cases and controls. Data were analysed with the Egret for Windows software (Version 1.0).

In the original study II the associations of serum enterolactone concentration with the risk factors for death were examined using covariate analysis. Serum enterolactone concentration was classified into 4 categories according to quartiles. These categories or serum enterolactone concentration as dummy variables were entered into forced Cox proportional hazards' regression models using SPSS 10.0 for Windows. Three different sets of covariates were used: the basic model included age, year of serum enterolactone measurement (2 categories) and the baseline examination years (1985, 1986, 1987, 1988, and 1989); (Multivariate model 1) included the basic model, diabetes, hypertension, urinary excretion of nicotine metabolites, BMI, alcohol consumption, serum LDL and HDL cholesterol; (Multivariate model 2) included Multivariate model 1, dietary intake of fibre, folate, vitamins C and E and saturated fatty acids. Their CI were estimated under the assumption of
asymptotic normality of the estimates. All tests for statistical significance were 2-sided. A stepwise linear multivariate regression analysis was used to find the strongest determinants of serum enterolactone concentration.

In the original study III the statistical significance of linear trend in plasma $F_2$-isoprostane over fifths of serum enterolactone was tested using one-way analysis of variance (ANOVA). A stepwise linear multivariate regression analysis was used to find the strongest determinants of plasma $F_2$-isoprostane level. Those were used as covariates in analysis of covariance to estimate the independent association of serum enterolactone with $F_2$-isoprostanes. Because the distribution of serum enterolactone values was skewed towards the higher end, enterolactone values were log-transformed to improve normality. All statistical analyses were repeated with the log-transformed enterolactone values but it had a minimal effect on the results. However, the value of R square for the dietary constituents accounting for the variation of enterolactone, was halved. SPSS for Windows statistical package (version 10.0) was used for all statistical analyses.

In the original study IV values were expressed as mean ± standard deviation (SD). The statistical significance of the heterogeneity of means across three treatment groups and differences between groups were tested with one-way ANOVA, entering the baseline serum enterolactone as a covariate. SPSS for Windows statistical package (version 10.0) was used for all statistical analyses.
5 RESULTS

5.1 Serum enterolactone concentration and the risk of acute coronary events (I)

5.1.1 Serum enterolactone concentration as a continuous variable

The mean (±SD) serum enterolactone concentration was 25.1 % lower among the cases (18.2 nmol/L±21.1) than among controls (23.5 nmol/L±18.2) (95 % CI, 4.1 to 46.2; P=0.001 for difference). In a univariate logistic model, the risk of acute coronary events was decreased on the average by 1.4 % per unit (nmol/L) of serum enterolactone (95 % CI, 0.2-2.6; P=0.016). The respective decrement of risk was 2.3 % per unit (nmol/L) of serum enterolactone (95 % CI, 0.7-3.7; P = 0.004) after adjusting for the nine most predictive risk factors, which were the same as shown in Table 5: model 2. The covariates were selected by stepwise analysis (P<0.05 for entry). Men with high serum enterolactone (above median, 15.1 nmol/L) had 52 % (95 % CI, 9.4-74.6; P=0.02) reduced risk of acute coronary events compared to men with lower serum enterolactone concentration.

5.1.2 Serum enterolactone concentration in quarters

To examine the dose-response relationship, the participants were divided into quarters of serum enterolactone concentration. Men in the highest enterolactone quarter (>30.1 nmol/L) had 59 % (95 % CI, 24.1-77.6; P= 0.005) reduced risk of acute coronary events as compared with the lowest quarter (Table 5: model 1, Figure 2). When this was adjusted for the nine most predictive risk factors (Table 5: model 2, Figure 2), men in the highest quarter had 65 % (95 % CI, 11.9-86.3; P= 0.03) reduced risk of acute coronary events as compared with men in the lowest quarter. The risk was not reduced significantly in the second lowest quarter of serum enterolactone concentration and was unchanged in the third quarter. The linear trend in acute coronary events risk across enterolactone quarters was statistically significant (P=0.01).
Table 5. Strongest risk factors for acute coronary events in conditional multivariate logistic regression models.

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>Model 1*</th>
<th></th>
<th>P</th>
<th>Model 2†</th>
<th></th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterolactone, fourth quarter (&gt;30.1 nmol/L)</td>
<td>0.41</td>
<td>(0.22-0.76)</td>
<td>0.005</td>
<td>0.35</td>
<td>(0.14-0.88)</td>
<td>0.03</td>
</tr>
<tr>
<td>Enterolactone, third quarter (15.1-30.1 nmol/L)</td>
<td>0.69</td>
<td>(0.31-1.57)</td>
<td>0.38</td>
<td>0.75</td>
<td>(0.31-1.81)</td>
<td>0.53</td>
</tr>
<tr>
<td>Enterolactone, second quarter (7.2-15.1 nmol/L)</td>
<td>1.11</td>
<td>(0.50-2.45)</td>
<td>0.80</td>
<td>1.22</td>
<td>(0.51-2.96)</td>
<td>0.51</td>
</tr>
<tr>
<td>Serum apolipoprotein B (per 100 mg/L)</td>
<td>1.31</td>
<td>(1.12-1.55)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary iron intake (mg/day)</td>
<td>1.12</td>
<td>(1.05-1.19)</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history of coronary heart disease (yes vs. no)</td>
<td>2.80</td>
<td>(1.40-5.66)</td>
<td>0.004</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic finding in the exercise test (yes vs. no)</td>
<td>3.23</td>
<td>(1.36-7.66)</td>
<td>0.008</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary calcium intake (per 100 mg/day)</td>
<td>0.89</td>
<td>(0.82-0.97)</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary excretion of nicotine metabolites (mg/day)</td>
<td>1.05</td>
<td>(1.01-11.10)</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (yes vs. no)</td>
<td>5.82</td>
<td>(1.36-24.98)</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (per 10 mmHg)</td>
<td>1.26</td>
<td>(1.02-1.54)</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal oxygen uptake (L/kg x min)</td>
<td>0.94</td>
<td>(0.88-0.99)</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A logistic regression model including three highest enterolactone quarters.
† A stepwise model (P for entry 0.05). The second and third enterolactone quarters are forced in to the model.

Figure 2. Unadjusted and risk factor adjusted odds ratios for acute coronary events according to quartiles of baseline serum enterolactone concentration.
5.1.3 Analysis of subgroups

5.1.3.1 Exclusion of men with subclinical CHD

To test the association between enterolactone and the risk of acute coronary events in participants with neither clinical nor subclinical CHD, 37 pairs were excluded with ischaemic electrocardiogram (ECG) in exercise test for either the case or the control. The association of enterolactone with the risk of acute coronary events was, if anything, stronger than for all pairs. For example, the unadjusted OR for the highest quarter of enterolactone was 0.33 (95% CI 0.14-0.76, P=0.01) as compared with the lowest quarter.

5.1.3.2 Comparison between smokers and non-smokers

Since smokers are at increased oxidative stress, the impact of antioxidative nutrients on CHD is expectedly greater in smokers. In an unadjusted unpaired logistic regression analysis in smokers, the relative benefit in the highest quarter was 79% (95% CI, 37.8-93.1; P= 0.005) and among non-smokers 47% (95% CI, -16.5 to 75.9; P= 0.11).

5.1.4 Association between serum enterolactone and blood pressure level

There was statistically significant heterogeneity in systolic and diastolic blood pressure between enterolactone quarters (Table 6). Men in the lowest enterolactone quarter had 4.7% (95% CI, 1.4 to 7.9%; P= 0.005) higher systolic blood pressure and 4.8% (95% CI, 1.7 to 7.8; P= 0.001) higher diastolic blood pressure than the men in the three highest enterolactone quarters. The means of total, HDL and LDL cholesterol and apolipoprotein B did not differ significantly between serum enterolactone quarters (Table 6).

5.1.5 Association with fibre

Serum enterolactone concentration had a weak positive correlation (r=0.08, P=0.15) with fibre intake. This correlation was stronger (r=0.12, P=0.03) when fibre intake was expressed per kilogram of body weight. The dietary intake of fibre had, however, no consistent association with the risk of acute coronary events.
Table 6. Blood pressure and serum lipids in quarters of serum enterolactone concentration from analysis of covariance.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1st quarter Mean±SE</th>
<th>2nd quarter Mean±SE</th>
<th>3rd quarter Mean±SE</th>
<th>4th quarter Mean±SE</th>
<th>P value for difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mmHg)*</td>
<td>142±1.9</td>
<td>136±1.9</td>
<td>134±1.9</td>
<td>137±1.9</td>
<td>0.026</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)*</td>
<td>93±1.2</td>
<td>89±1.2</td>
<td>88±1.2</td>
<td>90±1.2</td>
<td>0.017</td>
</tr>
<tr>
<td>Serum total cholesterol (mmol/L)‡</td>
<td>6.13±0.13</td>
<td>6.13±0.13</td>
<td>6.27±0.13</td>
<td>6.22±0.13</td>
<td>0.82</td>
</tr>
<tr>
<td>Serum LDL cholesterol (mmol/L)‡</td>
<td>4.25±1.13</td>
<td>4.34±1.03</td>
<td>4.39±1.02</td>
<td>4.19±0.99</td>
<td>0.52</td>
</tr>
<tr>
<td>Serum HDL cholesterol (mmol/L)‡</td>
<td>1.24±0.03</td>
<td>1.28±0.03</td>
<td>1.26±0.03</td>
<td>1.28±0.03</td>
<td>0.74</td>
</tr>
<tr>
<td>Serum apolipoprotein B (mg/L)‡</td>
<td>478±59</td>
<td>350±59</td>
<td>447±58</td>
<td>519±59</td>
<td>0.22</td>
</tr>
</tbody>
</table>

*Adjusted for age, examination year and alcohol intake, 24-hour urinary sodium excretion and dietary magnesium intake.
‡Adjusted for age, examination year and alcohol intake.

5.2 Serum enterolactone concentration and the risk of cardiovascular and all-cause death (II)

5.2.1 Baseline characteristics

The mean serum enterolactone concentration for the 1889 participants was 17.1 nmol/L (SD 14.0). Serum enterolactone concentration varied over 10-fold between the highest and lowest quartiles of the study population. Men with high serum enterolactone level were less often obese, hypertensive and smoked less. They also consumed less alcohol and more of fruits and berries and whole grain products and consequently their intake of water soluble vitamins was greater than in men with low serum enterolactone levels. Additionally, serum enterolactone concentration was higher among men with constipation.

5.2.2 Cumulative mortality

To illustrate the accumulation of the death cases according to serum enterolactone concentration we analyzed the data with Cox' proportional hazards' model adjusting for age, examination years and year of enterolactone measurement. In the Figures 6-8 the cumulative CHD-related, CVD-related and all-cause mortality respectively, are presented separately in
quarters of serum enterolactone to illustrate the earlier occurrence of deaths among men in the lowest quarters of serum enterolactone as compared to the others.

5.2.3 Serum enterolactone concentration and CHD and CVD-related mortality

In the Cox' proportional hazards' model low serum enterolactone concentration was associated with an increased risk of CHD and CVD-related mortality (Table 7). When serum enterolactone concentration was analyzed as a continuous variable and adjusted for age, and year of examination and enterolactone measurement there was a risk reduction of 17 % and 13 % for each 10 nmol/L of serum enterolactone in CHD (95 % CI, 0.69-0.99) and in CVD mortality (95 % CI, 0.75-1.00), respectively. The trend across serum enterolactone quartiles remained significant after adjustment for diabetes, hypertension, urinary excretion of nicotine metabolites, BMI, alcohol consumption, serum LDL and HDL cholesterol (P=0.029 for trend for CHD death and P=0.023 for trend for CVD death) and after adjusting for dietary factors such as dietary intake of vitamin C and E, folate, fibre and saturated fatty acids (respectively, P=0.034 for trend and P=0.042 for trend). In this multivariate model, men with high serum enterolactone level (≥23.9 nmol) had a 56 % reduced risk of CHD-related death (95 % CI, 0.20-0.96) and 45 % reduced risk of CVD-related death in the boarderline of significance (95 % CI, 0.29-1.01).

5.2.4 Serum enterolactone concentration and all-cause mortality

Serum enterolactone concentration, adjusted for age and year examination and of enterolactone measurement, was inversely associated with all-cause mortality (Table 7). When serum enterolactone concentration was used as a continuous variable and adjusted for the same covariates, there was a risk reduction of 12 % (95 % CI, 0.80-0.97) for each additional 10 nmol/L of serum enterolactone. After adjustment for diabetes, hypertension, urinary excretion of nicotine metabolites, BMI, alcohol consumption, serum LDL and HDL cholesterol the trend across the quarters of serum enterolactone concentration remained significant (P=0.046 for trend) but the risk reduction in the highest quarter (≥23.9 nmol) compared to the lowest (≤6.9 nmol/L) did not remain significant (RR, 0.72; 95 % CI, 0.49-1.05). Additional adjustment for diet weakened the observed association further.
Figures 6-8. Cumulative coronary (CHD), cardiovascular (CVD), and all-cause mortality, respectively in men according to quartiles (no. 1 indicating the lowest) of serum enterolactone concentration (nmol/L), adjusted for age, examination year and year of serum enterolactone measurement.
Table 7. Relative Risks (RR) of Coronary (CHD), Cardiovascular (CVD) and All-Cause Mortality According to Serum Enterolactone Concentration.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Quartiles of Serum Enterolactone Concentration</th>
<th>CHD-related Death</th>
<th>CVD-related Death</th>
<th>All-Cause Death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>No. of deaths</td>
<td></td>
<td>21</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td>Age and year of examination and of serum</td>
<td>1.00</td>
<td>0.89 (0.48-1.63)</td>
<td>0.73 (0.38-1.40)</td>
</tr>
<tr>
<td></td>
<td>enterolactone measurement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multivariate model 1*</td>
<td></td>
<td>1.00</td>
<td>1.13 (0.60-2.12)</td>
<td>0.89 (0.45-1.76)</td>
</tr>
<tr>
<td>Multivariate model 2†</td>
<td></td>
<td>1.00</td>
<td>1.14 (0.61-2.14)</td>
<td>0.94 (0.47-1.87)</td>
</tr>
<tr>
<td></td>
<td>Age and year of examination and of serum</td>
<td>1.00</td>
<td>0.85 (0.52-1.39)</td>
<td>0.61 (0.35-1.04)</td>
</tr>
<tr>
<td></td>
<td>enterolactone measurement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multivariate model 1*</td>
<td></td>
<td>1.00</td>
<td>1.01 (0.66-1.82)</td>
<td>0.80 (0.45-1.41)</td>
</tr>
<tr>
<td>Multivariate model 2†</td>
<td></td>
<td>1.00</td>
<td>1.13 (0.68-1.88)</td>
<td>0.85 (0.48-1.50)</td>
</tr>
<tr>
<td></td>
<td>Age and year of examination and of serum</td>
<td>1.00</td>
<td>0.88 (0.64-1.33)</td>
<td>0.64 (0.45-0.92)</td>
</tr>
<tr>
<td></td>
<td>enterolactone measurement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multivariate model 1*</td>
<td></td>
<td>1.00</td>
<td>1.05 (0.75-1.47)</td>
<td>0.82 (0.57-1.19)</td>
</tr>
<tr>
<td>Multivariate model 2†</td>
<td></td>
<td>1.00</td>
<td>1.07 (0.77-1.50)</td>
<td>0.85 (0.59-1.23)</td>
</tr>
</tbody>
</table>

*Adjusted for age, examination year, year of serum enterolactone measurement, diabetes, hypertension, urinary excretion of nicotine metabolites, body mass index, alcohol consumption, LDL and HDL cholesterol.

† Additionally adjusted for dietary intake of fibre, folate, vitamin C and E and saturated fatty acids.
5.2.5 Determinants of serum enterolactone concentration

The dietary constituent with the strongest univariate association with enterolactone concentration was energy adjusted intake of fibre which in a linear regression model explained ~6% of the variation of enterolactone. Of the factors that remained significant in the model (vegetable consumption, alcohol intake, saturated fatty acid intake, constipation, BMI and the number of bronchitis during lifetime) only vegetable consumption and constipation showed a positive association with serum enterolactone concentration. Other infections such as tonsillitis, sinusitis or ear infections were not associated with enterolactone. All of these variables together explained ~10% of the variation in serum enterolactone concentration.

5.3 Serum enterolactone concentration and lipid peroxidation (III)

5.3.1 Selection of variables

The mean serum enterolactone concentration was 16.6 nmol/L (range 1.1-70.8 nmol/L) and that for F2-isoprostanes 29.6 ng/L, ranging from 7.0 to 70.0 ng/L. The simple correlation coefficient for association between serum enterolactone concentration and F2-isoprostanes was −0.30 (P=0.003). Plasma F2-isoprostane levels increased linearly across serum enterolactone concentration (P<0.008 for a linear trend) (Figure 9). The unadjusted mean (95% CI) F2-isoprostane was 37.4 % greater in the lowest enterolactone fifth (<3.9 nmol/L) than in the highest fifth (>25.6 nmol/L).

In a linear regression model, the variables with the strongest associations with F2-isoprostanes selected by stepwise analysis (P in 0.05, P out 0.10) were alcohol consumption (standardised coefficient) 0.29, selenium intake -0.27, serum enterolactone concentration -0.24 and plasma tHcy concentration 0.20 (Table 8). Traditional risk factors such as LDL cholesterol, blood pressure, BMI and smoking assessed by 24 h urinary excretion of nicotine metabolites were tested for entry to the model but they had no residual association with F2-isoprostanes and were not selected. In the second model we forced the antioxidant vitamins with the strongest correlations with F2-isoprostanes to the model (Table 8). These were plasma α-tocopherol, β-carotene, ascorbic acid and dietary folate (energy standardised). Consequently the R square of the model rose from 37 to 39%. Serum enterolactone and other variables remained significant after this further adjustment with the antioxidant vitamins.
The strongest determinants of F₂-isoprostanes were also tested in a regression analysis separately in smokers (> 1 cigarettes per day) (n=49) and in non-smokers (n=51). In non-smokers, the associations of alcohol consumption (0.42, P=0.001), serum enterolactone concentration (-0.38, P=0.001) and selenium intake (-0.36, P=0.002) with F₂-isoprostanes were stronger, whereas plasma total homocysteine concentration (-0.03, P=0.8) showed no association.

5.3.2 Associations with dietary constituents

The dietary constituents with the strongest associations with serum enterolactone were water soluble fibre (r=0.39, P<0.001), water insoluble fibre (r=0.28, P=0.005) and intake of fruits and berries (r=0.25, P=0.01), vegetables (r=0.24, P=0.02) and cereals (r=0.19, P=0.06). Fibre components together in a regression analysis explained 17% of the variation of serum enterolactone whereas the other food groups mentioned explained 15% together. Of the dietary factors tested, only the intake of folate had a significant correlation with plasma F₂-isoprostanes (r=-0.21, P=0.04).

![Figure 9](image_url)

Figure 9. Mean plasma F₂-isoprostane concentration in fifths of plasma enterolactone concentration in 100 men from eastern Finland.
Table 8. The strongest associations of plasma F$_2$-isoprostane concentration (ng/L) in two multivariate models.

<table>
<thead>
<tr>
<th></th>
<th>4- variable model$^a$</th>
<th></th>
<th>8- variable model$^b$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstandardised</td>
<td>Standardised</td>
<td>P value</td>
<td>Unstandardised</td>
</tr>
<tr>
<td></td>
<td>regression coefficient</td>
<td>regression coefficient</td>
<td></td>
<td>regression coefficient</td>
</tr>
<tr>
<td></td>
<td>(95 % CI)</td>
<td></td>
<td></td>
<td>(95 % CI)</td>
</tr>
<tr>
<td>Serum enterolactone, nmol/L</td>
<td>-0.20 (-0.34--0.06)</td>
<td>-0.24</td>
<td>0.007</td>
<td>-0.18 (-0.33--0.03)</td>
</tr>
<tr>
<td>Energy adjusted selenium intake, µg/day</td>
<td>-0.19 (-0.31--0.07)</td>
<td>-0.27</td>
<td>0.003</td>
<td>-0.17 (-0.29--0.04)</td>
</tr>
<tr>
<td>Alcohol consumption, g/week</td>
<td>0.03 (0.01--0.05)</td>
<td>0.29</td>
<td>0.001</td>
<td>0.03 (0.009--0.50)</td>
</tr>
<tr>
<td>Plasma total homocysteine, µmol/L</td>
<td>0.79 (0.05--1.52)</td>
<td>0.20</td>
<td>0.04</td>
<td>0.85 (0.10--1.60)</td>
</tr>
</tbody>
</table>

$^a$ Variables with the strongest associations with F$_2$-isoprostanes selected by stepwise analysis.
$^b$ Additional adjustment for plasma alpha-tocopherol, beta-carotene, ascorbic acid and dietary folate.
5.4 Serum enterolactone concentration and phloem bread supplementation (IV)

5.4.1 Baseline characteristics

The baseline characteristics of the participants were as follows: age 50.7±10.9 years, BMI 25.8±2.7 kg/m², serum total cholesterol 6.8±1.0 mmol/L, systolic blood pressure 130±10 mmHg, diastolic blood pressure 83±9 mmHg and dietary intake of fibre 26±9 g/d. No significant changes were observed in any of these measured parameters (data not shown) and nobody reported of any side effects during the study period. According to results obtained by the two 4-day food record the participants’ compliance to given dietary and lifestyle instructions during the experiment was excellent. As all participants consumed fairly large amounts of rye bread on a regular basis most participants thought that the additional amount of rye bread made no difference to their normal diet.

5.4.2 Changes in serum enterolactone concentration

The mean serum enterolactone concentration at baseline was 31.5±27.5 nmol/L. Enterolactone concentrations at baseline between the three study groups differed somewhat from each other (P=0.052). These differences were not explained by fibre intake which was rather high for all but did not differ between the groups (P=0.79). No association was seen between serum enterolactone level and fibre intake (Pearson correlation coefficient 0.036).

Consumption of study breads increased the mean serum enterolactone concentration in all groups (by 1.9 nmol/L in placebo, 25.3 nmol/L in LP and 27.1 nmol/L in HP) (Figure 10). The variation of the increase between the groups was statistically significant (P=0.001). Analysis of covariance was performed in order to estimate the enterolactone concentration changes in the groups, taking the baseline differences into account. The heterogeneity of the enterolactone concentration change was highly significant across all three groups (P=0.005).
The increases of serum enterolactone concentration between the placebo and the LP group (P=0.009) as well as that between the placebo and the HP group (P=0.003) were statistically significant. The increase of serum enterolactone concentration did not differ between the LP and HP group (P=0.98). Substantial individual differences were observed in the response to study breads (Figure 11) and the ranges of enterolactone concentration changes in the groups were as follows: -54.5 to 60.0 nmol/L (placebo), -26.2 to 101.3 nmol/L (LP), -19.6 to 81.8 nmol/L (HP).

![Graph showing serum enterolactone concentrations at baseline and after supplementation](image)

**Figure 10.** Serum enterolactone concentrations at baseline and after the supplementation separately for all study groups.
Figure 11. Individual changes in serum enterolactone concentration during the study in the three study groups.
6 DISCUSSION

6.1 Serum enterolactone concentration as study variable

For the measurement of serum enterolactone concentration in all the presented studies a time-resolved fluoroimmunoassay method (TR-FIA) was used. This method combines the advantages of other non-radioisotopic assays with a 10-100-fold increase in sensitivity and assay range in comparison with conventional enzyme-immunoassay and fluoroimmunoassay methods. The results of plasma enterolactone concentration obtained by TR-FIA are comparable to those obtained by GC-MS method, the specificity of which makes it valuable as a reference method (132). Previously used analytical methods for detection and quantification of lignans in human biological fluids are based on GC or HPLC in combination with MS. These expensive and time-consuming methods are not suitable for screening purposes in large populations. To our knowledge, the original study I was the first study were serum enterolactone concentrations were measured in a population based sizeable sample.

In the original studies I and II the serum samples drawn at baseline were stored at -20°C for 11.9 and 13.4 years, respectively. It could be argued that the long storage time might affect the results obtained by TR-FIA and thus present a source for a possible bias. Interestingly though, comparing the mean serum enterolactone concentration values between the different samples of Eastern Finnish men in the original studies II and III, where in the latter the serum samples were stored for 4 years at -70°C prior to analysis, only a difference of 0.2 nmol/L was observed. The possible bias due to analysing the cohort’s (n=2557) enterolactone concentration in two parts in the original study II (the case-control data in 1998 and the rest in 2000) was accounted for in the statistical analysis. This was done by creating a variable indicating the year of measurement, which was then included in all the Cox’ proportional hazards’ regression models. All serum enterolactone analyses were carried out in the same laboratory and under the guidance of the same supervisor.
6.2 Serum enterolactone concentration and acute coronary events, CHD, CVD and all-cause mortality (I & II)

6.2.1 Methodological aspects

In the case-control setting of the original study I, the association of serum enterolactone concentration with the risk of acute coronary events or with CVD altogether, was investigated for the first time. After expanding the serum enterolactone analyses to cover the entire cohort baseline, serum enterolactone concentration was studied in relation to CHD, CVD and all-cause mortality risk reported in the original study II. In previous studies serum enterolactone concentration has not been associated with mortality.

The possibility to measure the exposure variable from serum, involves some benefit in comparison to the inaccuracy derived from several diet recording methods. On the other hand, at the same time the data on the exposure variable is limited on a narrow time span. This might be a problem in the case in which the production of enterolactone has temporarily been discontinued due to the effects of antibiotic treatment on intestinal bacteria, which can result in lowering the concentration in serum. Unfortunately, in the original studies I and II the data on the use of antibiotics during the previous months was not available and thus could not be controlled for. Even although one measurement result predicts relatively well the level of serum enterolactone over two year period at population level (84), among individuals the effects of dietary changes on serum enterolactone concentration are significant. Due to these aspects, multiple measurements of serum enterolactone concentration during the follow-up period would have been preferable. On the other hand, a random measurement variability in the exposure variable tends to attenuate the observed associations with disease risk.

The design in the original studies I and II provided an opportunity to investigate the predictive value of a single measurement of serum enterolactone concentration and regarding the risk of acute coronary events, CHD and CVD mortality and all-cause mortality. The prospective design in the original studies I and II also allows some assumptions of the temporality of the association to be made although any speculation of causality should be done cautiously. The large representative population-based sample of middle-aged men supports the generalizability of the observed findings to other male populations. However, the distinct dietary and other health related behaviour of the Eastern Finnish men might be important to consider. The relatively long follow-up time provided a sufficient number of acute coronary events and of CHD, CVD and all-cause death cases to assure the statistical
power of the analysis. Additionally, a substantial advantage is that at the cohort baseline a large number of possible and potential risk factors were measured to ensure the ability to control for confounding. However, the concern that some other unconsidered factors affect the results by residual confounding cannot be entirely excluded.

6.2.2 Results

In the nested case-control study (I) we observed a clear reduction in the risk of acute coronary events among men with high serum enterolactone concentration. The number of acute coronary events was sufficient for reliable analyses of the data and this finding persisted after the adjustment for the nine most strongly predictive risk factors. In the cohort study (II) we observed a clear reduction in CHD-related, CVD-related and all-cause mortality in men with high serum enterolactone concentration. The associations with CHD and CVD mortality persisted after the adjustment for potentially confounding variables as well as for diet but regarding all-cause death the adjustment for diet weakened the association considerably.

In the original study I, among smokers the high serum enterolactone concentration was even of greater benefit. Partly due to the enhanced lipid peroxidation caused by cigarette smoke, smokers are under higher oxidative stress (133-135). Consequently, the benefit gained by increased antioxidant intake can be expected to be higher (136). This discrepancy among smoking status in connection with enterolactone could be an implication of antioxidant action, if not of enterolactone itself then something in association with it.

Since mid eighties, when the collection of dietary data in the study cohort was started, major changes in dietary behaviour in Finland have occurred. By far the greatest change has been the steep decline in the consumption of liquid milk products that has started already in the beginning of seventies. Changes that might affect the predictive value of a single measurement of serum enterolactone concentration have also occurred. Of these changes the decline in the intake of grain products and the increase in vegetable and fruit consumption are probably most important. Still even today Eastern and Northern Finland are distinguished by their higher bread, particularly rye bread consumption which explains also their higher fibre intake compared to the rest of Finland (137, 138). Consequently it can be speculated that the decline in the consumption of grain products has not been as steep in the Eastern parts and this might partly explain why the level of serum enterolactone still predicts the risk of CVD after eight to twelve years of follow-up.
The inverse association between serum enterolactone concentration and blood pressure observed in both the nested case-control (I) and in the cohort setting (II), could be explained by the effects of a balanced diet and moderate alcohol consumption on blood pressure levels. Men with higher serum enterolactone concentration consumed more fruit and vegetables, grain fibre, potassium and less saturated fat and sodium, which are likely to influence blood pressure levels. These dietary factors are associated with other lifestyle factors such as physical activity and socio-economic class that might explain some of the observed effect. With the evidence available, it is difficult to speculate about the role of enterolactone in this setting.

Unfortunately, we were unable to show a strong association between serum enterolactone and dietary intake of fibre or with any other dietary component. This finding underlines the importance of other determinants like the intestinal microflora, which has a central role in the lignan metabolism. Intestinal bacteria are subject to large individual differences, possible due to genetic variation (139) and even the effects of diet on the consistency of the intestinal bacteria are poorly understood. Dietary data in the studies were collected by a 4-day self-monitored food consumption record, the characteristics of which as a dietary assessment tool may be discussed. Filling the dietary record sheets for several days is demanding for the study participants, but it usually provides relatively accurate information on the dietary pattern compared to several other methods. The food record data were also collected close (within eight days prior) to the collection of the blood samples. Anyhow, some criticism is also to the point. Reporting bias might be a relevant concern in assessing dietary data as well as changes in dietary behaviour that have occurred during the days of the self-monitoring. Thus, the four days of which the dietary data are available might not represent the usual diet. Naturally, the possibility of having even more accurate dietary data by e.g. collecting four-days food consumption records at all four seasons would have been advantageous.

6.3 Serum enterolactone concentration and lipid peroxidation (III)

6.3.1 Methodological aspects

The cross-sectional data from the 100 men at the ASAP study baseline represents a reasonable size study to look for an association between serum enterolactone concentration and lipid peroxidation. Sample included high CVD risk males, which in respect to homogeneity, adds to the internal validity. Half of the participants were smokers, allowing analysis to be made,
also separately, according to smoking status. \( F_2 \)-isoprostane (8-\textit{epi}-PGF\(_2\alpha\)) concentration that results from the oxidative modification of arachidonic acid is considered as fairly reliable marker of the free-radical attack of cell membrane phospholipids or circulating LDLs as a measure of lipid peroxidation (45). Despite of being only minor products of the peroxidation process, compared to several other assays measuring different lipid peroxidation products, isoprostane analysis fulfills many of the unofficial criteria set for a reliable biomarker of lipid peroxidation (16). To facilitate the determination of \( F_2 \)-isoprostanes several kits based on ELISA (enzyme linked immunosorbent assay) have become commercially available but until now they have not reached the reliability of the GS-MS method used in this study. Thus, the analyzing method is important to consider when comparing results from different studies. It has been criticised that the values of \( F_2 \)-isoprostanes could be influenced by plasma arachidonic acid or even dietary arachidonic acid (140). These arguments, however, have not been proven (43) and thus the discussion is still ongoing. Unfortunately, the cross-sectional study setting does not allow the consideration of temporality of the associations, which restricts the speculation of the causal connection between serum enterolactone concentration and lipid peroxidation. Our analysis of \( F_2 \)-isoprostanes was based on the level of a single fasting blood specimen and which might not represent the habitual level of lipid peroxidation. According to a metabolic study of \( F_2 \)-isoprostanes on rabbits the plasma elimination half-life, only traces were left after 20 minutes of administration (141). The implication of this result in humans could be speculated but undoubtedly several measurements of plasma \( F_2 \)-isoprostanes or 24-hour urinary excretion of \( F_2 \)-isoprostane would have represented more reliably the level of lipid peroxidation over prolonged time span.

### 6.3.2 Results

In the original study III, plasma \( F_2 \)-isoprostane concentration decreased linearly across fifths of serum enterolactone concentration. This was an interesting finding as the previously suggestive evidence was available only from \textit{in vitro} studies. The persistence of serum enterolactone concentration as a predictive factor of lipid peroxidation even after the adjustment for plasma \( \alpha \)-tocopherol, \( \beta \)-carotene, ascorbic acid and dietary folate, supports the idea of the existence of other possible less familiar but potentially important dietary determinates of oxidative stress. These may include a variety of phenolic substances of which lignans represents just one group of compounds. Among smokers lipid peroxidation seemed to be explained solely by homocysteine. Although in the original study I, smokers were shown to gain additional benefit from high serum enterolactone concentration, the results in
the original study III do not support the concept, that in smokers the benefit of high enterolactone concentration would be mediated through its effects on free radical stress. However, the sample size of 49 smokers reduces the sense in further speculation.

6.4 Serum enterolactone concentration and phloem bread supplementation (IV)

6.4.1 Methodological aspects

To detect changes in serum enterolactone concentration, the 4-weeks supplementation period can be considered as a sufficient period to detect a change. Participants were instructed to change their usual diet as little as possible despite the additional rye bread. Four-day food recordings were carried out at baseline and during the supplementation period to detect any major changes in the diet but naturally this does not equal up to the optimum situation where only a single component of diet is under a change. Thus, we cannot fully rule out the possibility that something else than the supplementation e.g. changes in the intake of other dietary constituents have affected the observed effects.

6.4.2 Results

The results from previous supplementation studies show the elevating effect of lignan containing foodstuffs on serum enterolactone (70, 72, 90). Often the lignan content of the used foodstuffs has been known only partially. This has lead to the inconsistent results where the yield of enterolactone has exceeded that of its dietary precursors (72). Our collaborator, Fölkhälsoan Research Center’s laboratory provided us the lignan analysis of the study bread, including the quantitative results of the newly found lignans (pinoresinol, lariciresinol, syringaresinol). These analyses gave an extended picture of the variety of lignans in the study bread.

In this study, clear individual changes in serum enterolactone following the phloem fortified rye bread supplementation could been seen. This is the first publication in which the individual response of lignan intake on serum enterolactone concentration has been described in a relatively large study. This evidence demonstrates clearly the major impact of additional factors influencing serum enterolactone concentration, other than the diet. The limitation of the study was that the diet could not be controlled for. Unfortunately, we did not have the opportunity to measure F₂-isoprostanes in this study in order to obtain evidence of the possible causal relationship between lignan intake and lipid peroxidation.
7 SUMMARY AND CONCLUSIONS

The results of this thesis can be summarised as follows:

1. High serum enterolactone concentration was independently associated with a decreased risk of acute coronary events.
2. High serum enterolactone concentration was protective against premature CHD-related, CVD-related and all-cause death.
3. Low serum enterolactone concentration was a significant determinant of enhanced lipid peroxidation, measured as plasma F₂-isoprostanes.
4. Supplementation of phloem powder fortified rye bread elevated serum enterolactone level, although there were significant individual differences in the response.

Based on the evidence presented by these studies it can be concluded that a high serum enterolactone concentration can predict a reduced risk of coronary events. Also, it appears to be inversely associated with cardiovascular and all-cause mortality in eastern Finnish men. These observations are in agreement with the findings suggesting whole grain fibre, fruit and vegetables to be protective against cardiovascular disease. The associations observed between serum enterolactone concentration and cardiovascular disease and lipid peroxidation can also reflect the importance of dietary lignans or other associated compounds. Intestinal bacteria, which are essential in the metabolism of many polyphenols can have a significant role in the prevention of chronic disease.
8 FUTURE DIRECTIONS

1. Prospective epidemiological studies in different populations are needed to confirm and to further investigate the role of lignan intake and enterolactone and in the prevention of CVD and other diseases. Finally, clinical trials are warranted to establish the causal connection between lignans and disease risk.

2. The future research developments should include specific biomarkers of many dietary polyphenols, which together with complete database of dietary polyphenols will improve the measure of exposure.

3. With the advances in biotechnology researchers have the possibility to take into account more specifically constitutional and other individual characteristics and tendencies. This might increase our understanding of individual differences in the antioxidant defence system and divergences in the intestinal flora composition.
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